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Adeno-Associated Viral Vector-Driven Expression of Coagulation Proteins for Treatment of Hemophilias and Cancer

Julie Marie Crudele

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Abstract

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The second part of the dissertation attempts to determine the underlying mechanism of the known anti-metastatic effects of activated protein C (aPC). We again utilized liver-directed AAV gene therapy to tease apart the anticoagulation and cytoprotective effects of aPC, as these were the most likely candidates based on available literature, in the B16F10 murine model of metastatic melanoma. Upon finding, however, that neither of these functions were involved in aPC's cancer protection and that zymogen PC is even more protective, we endeavored to find the novel mechanism through which the protein C pathway can modulate tumor progression. While the mechanism has not been found, a number of potential candidates and receptors have been eliminated, including involvement of PAR-4, EPCR, and a potential integrin binding site. We also demonstrate that it is not tumor growth, but rather a step in the metastatic pathway that is inhibited.

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ADENO-ASSOCIATED VIRAL VECTOR-DRIVEN EXPRESSION OF
COAGULATION PROTEINS FOR TREATMENT OF
HEMOPHILIAS AND CANCER

Julie M. Crudele

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CANCER

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Julie Marie Crudele

For my father,
who always encouraged me to “have fun and cure cancer.”

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ABSTRACT

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CHAPTER 1

INTRODUCTION

I. Adeno-associated viral vector gene therapy

One in every 200 live-born babies will have a monogenetic disorder—such as cystic fibrosis, hemophilia, or sickle cell anemia—of which there are more than 6000 known diseases.¹ Many more will be affected by polygenic and/or environmentally-influenced pathologies, including Alzheimer's disease, heart disease, diabetes, arthritis, and cancer. Unfortunately, treatment options are not always available or successful for the wide-range of genetic diseases. When therapies do exist, they can involve life-long and often expensive pharmaceutical, surgical, and/or life-style interventions and are usually focused on ameliorating the consequences of the genetic defect, rather than correcting the underlying problem.

Gene therapy is a potential treatment for many of the monogenetic diseases where the culprit gene has been identified and for multifactorial inherited and acquired disorders where a known gene product would help ameliorate disease phenotype. Regulation of expression, correction or *de novo* expression of a gene is achieved by packaging the required therapeutic transgene cDNA into a delivery vector—often a virus, liposome, or plasmid DNA, which is used to transduce target cells. This can be done directly by *in vivo* delivery of vector or indirectly *ex vivo*, in which cells are harvested, transduced *in*

vitro and then returned to the patient. Given that the treatment is typically meant to lead to production of a novel protein, there is a risk of an inadvertent immune response against the transgene and the targeted cells, especially in instances where the underlying genetic mutation requiring correction is an early stop codon, frame shift, or large inversion or deletion. With missense mutations, conversely, patients are often already tolerized to most—if not all—of the protein epitopes, so the risk of an immune response is diminished. Furthermore, there is also the possibility of a reaction against the vector, which can lead to destruction of the transduced cells², flu-like symptoms³, cytokine storm, and even death⁴. Thus, vectors must be chosen carefully based on the desired target cells and intended purpose, with consideration given to potential negative outcomes.

Adeno-associated virus (AAV)⁵ is a small, non-enveloped parvovirus (family *Parvoviridae*, genus *dependovirus*) that has been isolated from numerous species, including humans. It has a 4.7-kb, single-stranded DNA genome that converts to double-stranded DNA after infection.⁶ The relatively small genome consists of only two genes encoding replication (*rep*) and capsid (*cap*) proteins, framed by inverted terminal repeats (ITRs). With so few genes encoded, AAV is naturally replication defective and requires the machinery of a “helper” virus such as an adenovirus or herpes virus to advance to the lytic stage.⁵ In the absence of such help, wild type AAV persists in a latent state by integrating into the human host genome, usually at a specific site on chromosome 19.^{7,8} This integration is rep-directed, however; so in its recombinant vector form—with *rep*

and *cap* deleted—the genome persists as concatemers in an extra-chromosomal state.⁸

The rarity of integration events with AAV vectors is associated with a favorable safety profile by decreasing the risk of tumor-suppressor disruption or oncogene activation, both of which can lead to cell transformation and cancer. However, persistence in dividing cells is limited by its episomal state, and thus AAV is best suited for transduction of postmitotic cells when long-lasting expression is sought. AAV is not known to cause any illnesses in humans, despite a high prevalence of seropositivity in humans.⁹ Therefore, recombinant AAV is a promising gene therapy vector due to its nonpathogenic nature, ability to transduce non-dividing cells, serotype-dependent targeting of a wide variety of cell types, and long-term genomic persistence in non-dividing cells.

There are two major limiting factors to AAV's potential efficacy as a gene therapy vector. The first is the limited packaging capacity. By most counts, AAV vectors cannot efficiently package more than 5 kb of DNA¹⁰, which greatly limits the transgenes, promoters and enhancers that can be used. Secondly, while minimally immunogenic and with involvement of the innate immune system limited mostly to TLR-9^{11, 12, 13}, there are still adaptive anti-AAV immune responses that can limit efficacy. Specifically, anti-AAV antibodies can inhibit primary *in vivo* transduction¹⁴ and prevent vector re-administration, while anti-AAV capsid cytotoxic T cells can lead to destruction of transduced cells². Approximately 90% of humans are seropositive for at least one serotype of AAV⁹, which limits the potential patient population. However, prevalence of

neutralizing antibodies (NAb) varies greatly with serotype: anti-AAV2 NAb are amongst the most prevalent because humans are the natural AAV2 host, while anti-AAV5 and 8 are amongst the least¹⁵. Unfortunately, anti-AAV2 NAb can cross react with a number of other serotypes. The cytotoxic CD8⁺ T cell responses to AAV capsid that can lead to killing of transduced cells have been demonstrated in multiple gene therapy clinical trials following intravascular or direct intramuscular injection of the vector^{16, 17}. To date, limited experience with intravascular delivery of AAV has shown that capsid-associated immunogenicity is transient and lasts only until a relatively rapid vector capsid clearance, is vector-dose dependent, and is potentially controlled with a short course of mild immunosuppression. Interestingly, sub-retinal injection of AAV vectors in early phase clinical trial is not influenced by the presence of NAb to AAV capsid, even for vector re-administration, or by the cytotoxic T cell responses^{18, 19, 20}. This is probably due to the immune privileged status of the eye.

Despite these challenges, adeno-associated viral vector gene therapy has the potential to treat a wide range of monogenetic and multifactorial diseases.

II. Hemophilia and the immune system

Hemophilia A and B, monogenetic diseases and the focus of the second and third chapters, are caused by mutations in the X-linked genes encoding clotting factor (F) VIII or IX, respectively. Patients are classified based on the severity of disease²¹ (**Table 1.1**), which is directly related to the residual clotting factor activity, with patients with severe

hemophilia retaining less than 1% of normal activity levels of FVIII or FIX. Notably, an increase in sustained activity levels to just above 1% can have a profound impact on the disease phenotype and a patient's quality of life, with levels over 5% almost entirely ameliorating day-to-day symptoms. Mild and moderate hemophilia are usually caused by missense or late nonsense mutations, while large deletions, rearrangements, early stop codons or frame shifts, partial duplications, or inversions typically result in severe disease. The most common mutation, affecting up to 40% of patients with severe HA, is an inversion breaking intron 22 and reversing the first 22 exons of FVIII²². Patients with the intron 22 inversion or some of the other severe mutations have no circulating FVIII or FIX, depending on the affected gene, in their bloodstream (described as negative for cross reactive material; CRM-).

1 in 5,000 males born worldwide—independent of race—will suffer from hemophilia A (HA), while hemophilia B (HB) affects 1 in 30,000 males. Current standard-of-care treatment for hemophilia consists of replacing the missing or mutant factor via

Table 1.1 Hemophilia severity classifications

Severity classification	Residual factor activity, % of normal	Common phenotype	Bleeding frequency	Prevalence
Severe	<1%	Spontaneous bleeding; joint bleeding	Weekly	~60-70% of HA ~50% of HB
Moderate	1-5%	Bleeding with slight injury; may have joint bleeding	Monthly	~15% of HA ~30% of HB
Mild	>5%	Bleeding with major trauma or surgery; rarely has joint bleeding	Potentially never	~25% of HA ~20% of HB

intravenous infusions of wild type protein concentrates given on-demand in response to bleeds or, to patients in the developed world, prophylactically. Sources of factor include recombinant protein and protein purified from pooled donor plasma.²¹ In the United States, where the standard of care is prophylactic administration of factor, the cost of care for a single hemophilia A patient is about \$200,000 per year depending on the therapeutic regimen and body weight of the patient.²³

A major treatment complication facing hemophilia patients in the industrialized world today is the development of polyclonal antibodies, clinically termed inhibitors, that block the therapeutic activity of the infused factor. Inhibitors will develop in about 20-30% of individuals with severe HA and 5-10% of individuals with mild or moderate HA, while inhibitor formation is closer to 1-3% of individuals with HB^{24, 25}. While it is not currently possible to predict with certainty which patients will develop inhibitors^{26, 27, 28}, there are associated risk factors. Underlying mutation correlates strongly with risk. For HA, incidence of inhibitors is greater than 30% in patients with large deletions, nonsense mutations, and large inversions, but only 8% in patients with missense mutations²⁹. Additionally, the chance that a patient will develop inhibitors increases when he is of non-Caucasian descent, has a family history of inhibitors, is first exposed to factor concurrently with surgery or trauma, or is treated on-demand rather than prophylactically^{28, 29, 30}. Polymorphisms in the TNF- α and CTLA-4 genes and the IL-10 promoter are also associated with higher risk of inhibitors in HA (no such data exists for HB due to

limited patient numbers).^{31, 32, 33} In severe hemophilia, onset usually occurs within the first 20 factor infusions.^{26, 28}

Patients with inhibitors are grouped based on inhibitor titer (defined as Bethesda Units, BU, where 1 BU inhibits 50% activity of normal plasma) and treated accordingly. Those with >5 BU are classified as high-responders while those with 0.6-5 BU are low-responders; <0.6 BU is considered negative for inhibitors.³⁴ Low-responders are treated with higher or more frequent doses of factor in order to overwhelm the circulating antibodies and establish hemostasis. In high-responders, antibodies can be temporarily removed from the plasma by plasmapheresis to allow for treatment with factor. This, however, is a short-term solution reserved for life-threatening situations.^{34, 35} Instead, hemophilia patients with high-titer inhibitors in wealthy countries are typically treated with bypass agents, including activated FVII or activated prothrombin complex concentrates, which circumvent the need for FVIII or FIX in the clotting cascade. Unfortunately, bypass agents, which are extremely costly, are not as efficacious as FVIII/FIX replacement at alleviating bleeding³⁶, so patients suffer from higher joint damage, lower quality of life, and shorter lifespans^{34, 37, 38, 39, 40}. Thus the presence of inhibitors is associated with both increased morbidity and mortality.

The best course of action for inhibitor patients is tolerization to FVIII or FIX through intensive immune tolerance induction (ITI) protocols. ITI is effected through frequent (typically daily), uninterrupted factor infusions^{41, 35, 42}, sometimes coupled with

immunosuppression for HB patients refractory to ITI^{43, 24} despite the lack of evidence that this increases success rates, followed by enduring prophylactic infusions once tolerization is established to prevent anamnestic responses upon re-exposure to FVIII or FIX concentrate. The average time to tolerance induction in HA ranges from 4 to 12 months, but 48 months of treatment is required for 90% of HA patients to be successfully tolerized^{26, 27}. ITI success rates vary from study to study, usually ranging from 55-80% for HA and 10-60% for HB, with high-responders requiring longer periods on ITI and demonstrating higher rates of failure. Patients are more likely to be tolerized if they have had fewer factor infusions between the time of inhibitor development and initiation of ITI, lower titer inhibitor at the time of ITI initiation (<10 BU), and lower peak inhibitor titer^{26, 27, 44, 41}. Additionally, in the case of FIX inhibitors, ITI carries a risk of anaphylaxis and nephrotic syndrome, both of which decrease the likelihood of successful retolerization.²⁴ It costs approximately 1 million US dollars to tolerize patients through high-dose, frequent factor infusion⁴⁵, though this is significantly less than the cost of care via life-long bypass agents. Fortunately, relapse rates for HA are <5%²⁷.

The dependency of inhibitor development on CD4⁺ T cells has been shown in hemophiliac patients with advanced AIDS whose inhibitor titers fell with CD4⁺ T cell counts⁴⁶ and experimentally in mice demonstrating CD4⁺ T cell proliferation during onset of inhibitors⁴⁷. Additionally, inhibitor development in mice was prevented and pre-existing inhibitor production diminished when CD4⁺ T cell activation was blocked by knocking out B7-2 or treating with CTLA4-Ig⁴⁸. While these studies indicate that

inhibitor development is CD4⁺ T cell dependent, not much else is known about the mechanisms through which they arise.

Furthermore, despite over 30 years of clinical practice, the underlying mechanism by which ITI is successful remains incompletely understood and is likely to be complex.

One study established that high-dose FVIII infusions in HA mice with inhibitors leads to irreversible inhibition of FVIII-specific memory B cells in a T cell-independent fashion⁴⁹. While this was an important discovery explaining ablation of existing FVIII responses during ITI, it does not explain how tolerance is maintained long-term. In humans, Tregs have been implicated by an in vitro study in which CD4⁺CD25⁺ T cell-depletion of PBMCs from healthy individuals resulted in an enhanced CD4⁺ T cell proliferative response to FVIII⁵⁰. Additionally, CD4⁺ T cells isolated from the peripheral blood of a mild HA patient with inhibitors were more responsive after stimulation with FVIII upon Treg depletion⁵¹.

There is evidence that, in the right setting, gene therapy can actually induce immunological unresponsiveness to a therapeutic protein, with the advantage that the protein can be continuously expressed, rather than the stop-and-start levels provided by pharmaceuticals. For example, liver-directed gene therapy utilizing AAV vector can effectively tolerize animal models against the transgene^{52, 53, 54}. The mechanisms of tolerance in this setting have been well studied in mice, involving CD4⁺ T helper cell clonal deletion, anergy, and CD4⁺CD25⁺FoxP3⁺ Treg-mediated suppression^{52, 55, 56, 57}.

However, data elucidating the mechanisms of tolerance by gene therapy in large HA animal models and by intravenous protein infusion in humans is lacking.

III. Cancer metastasis and coagulation

It has long been known that cancer and coagulation are inextricably linked. Cancer patients are at a higher risk of developing thrombosis, and unprovoked venous thromboembolism (VTE) can actually be an indicator of undiagnosed cancer in some cases^{58, 59}. The mechanisms through which malignant neoplasms initiate coagulation are debated and likely numerous and variable. There are data supporting tumor-derived tissue factor (TF)^{60, 61, 62}, factor X (FX)-activating cysteine proteinases^{60, 63}, and platelet-activating mucins^{60, 64}, among others⁶⁰, as potential mechanisms of cancer procoagulation.

In addition to cancer causing thrombosis, the corollary is also true: procoagulant status enhances tumor metastasis. Incubating cancer cells with soluble fibrin⁶⁵ or thrombin^{66, 67} prior to injection into murine models enhance their metastatic potential, and mice with the procoagulant factor V (FV) Leiden variant have more metastases.⁶⁸ It logically follows, therefore, that metastasis can be reduced by downregulation, deficiency and inhibition of various coagulation pathway players—from factors like TF^{69, 70, 71, 72, 73, 74}, prothrombin^{69, 75} and fibrinogen^{69, 75, 76, 77} to receptors such as PARs^{69, 77, 78} and P-selectin^{69, 79, 80, 81} to even platelets^{69, 82, 81, 77}. Likewise, hemophiliac mice develop fewer metastatic lesions compared to wild type mice⁶⁸.

The exact mechanisms through which the coagulation cascade contributes to metastatic disease are debated, but there are data supporting a number of hypotheses, suggesting it is multi-faceted. Clots facilitate cancer cell spreading and endothelial adhesion^{69, 83}, partially by giving the cancer cells a surface to adhere to and partially by slowing down the flow of blood and protecting against shear stress^{84, 69}. Platelet and endothelial PARs and P-selectins^{69, 81} might provide necessary extravasation receptors, and recruited platelets^{69, 85}, monocytes and macrophages^{69, 86} can promote tumor cell growth and survival. Protecting the nascent tumors from destruction by the immune system is equally important; clots can physically block natural killer (NK) cells from accessing tumor cells for lysis and induce NK quiescence.^{69, 87, 88, 82, 75, 89, 90}

With such overwhelming evidence that coagulation enhances cancer progression, therapies targeting the coagulation cascade have been explored for their ability to prevent cancer progression in animal models. Coumadin (warfarin) reduced the number of lung tumors formed in rats injected intravenously with cancer cells^{69, 91}, as did hirudin in mice^{69, 92, 93}. Anticoagulants have also tentatively proven to be beneficial in cancer patients. Low molecular weight heparin has been shown to confer a survival benefit to cancer patients^{94, 95}, although not consistently⁹⁶, and the role of warfarin in preventing tumor formation or metastasis in humans is debatable. Daily aspirin reduced disease progression⁹⁷ and death⁹⁸ in patients with prostate cancer, breast cancer⁹⁹, and other cancers¹⁰⁰, and reduced the frequency of disease recurrence in patients with colorectal

cancers^{101, 102}. Of course, aspirin has multiple effects beyond just anti-coagulation, including reducing inflammation, which may be at least partially responsible for the reduced risk of cancer progression.

Activated protein C (aPC)—the focus of Chapters 4 and 5—is another anti-coagulant that has been shown to have protective effects in animal models of cancer^{103, 104}. aPC is a vitamin-K dependent, serine protease glycoprotein that circulates in plasma as a zymogen (zyPC), *i.e.* an inactive enzyme precursor. The human protein C (hPC) *PROC* gene is found on chromosome 2 and encodes the 419 amino acid proenzyme¹⁰⁵, which consists of a phospholipid-binding Gla domain, an aromatic stack segment, two epidermal growth factor (EGF)-like domains, an activation peptide, and a protease domain¹⁰⁶. Most zyPC circulates as a heterodimer consisting of a heavy chain (the activation peptide and the protease domain) and a light chain (the Gla domain, aromatic stack, and EGF-like domains). Cleavage at arginine 169 in the heavy chain by thrombin releases the activation peptide, converting the zymogen to aPC. This cleavage event requires thrombin to be bound to its transmembrane cofactor, thrombomodulin (TM), on the endothelium and is kinetically disfavored unless zyPC is bound to its endothelial protein C receptor (EPCR)¹⁰⁶.

EPCR (CD201) is a non-signaling transmembrane protein with homology to the CD1/major histocompatibility complex superfamily^{107, 106} expressed on the surface of endothelial cells^{106, 107, 108} as well as NK cells, monocytes, neutrophils and eosinophils.

Expression on the endothelium is uneven, with higher levels found on the surface of larger vessels^{109, 110}. EPCR is bound by zyPC and aPC with equal affinity^{111, 112} at a K_D that indicates, based on hPC circulating plasma levels, that the majority of EPCR should be occupied by PC¹¹³. This makes PC an anticoagulant “on demand,” ready and waiting to be activated by thrombin.

Activated protein C’s anticoagulant function is mediated through its protease domain. Upon activation by the thrombin-TM complex, aPC can dissociate from EPCR and localize to the phospholipid bilayer on cell surfaces, where aPC irreversibly cleaves activated factors V and VIII (FVa and FVIIIa), leading to their inactivation¹¹⁴. It is worth noting that FVIIIa is inherently unstable, and whether physiologic levels of aPC will actually cleave FVIIIa prior to spontaneous inactivation via dissociation of the A2 domain is debated¹⁰⁶. Cleavage of FVa is greatly enhanced by PC’s non-enzymatic cofactor, protein S, while cleavage of FVIIIa is only moderately improved^{106, 115}.

Activated protein C also has anti-inflammatory and cytoprotective effects on the endothelium affected by EPCR and the G-protein coupled receptor PAR-1 (protease-activated receptor 1). PAR-1 signaling is initiated when an extracellular N-terminal cleavage event produces a new N-terminus, which acts as a tethered ligand and results in outside-in signaling^{109, 116}. Notably, this signaling can result either in the aPC-mediated anti-inflammatory and cytoprotective effects or in thrombin-mediated pro-inflammatory and barrier disruptive effects depending on the context in which the cleavage occurs.

There is contention in the field as to whether the cytoprotective cleavage is directly by aPC (upon activation, a large portion—at least 30%—of aPC remains bound to EPCR^{113, 117}), which can perhaps cleave PAR-1 at a unique site and thus produce a novel tethered ligand responsible for the differential signaling¹¹⁸. Alternatively, disruption of receptor clustering in caveolae of PAR-1 with EPCR due to aPC-EPCR binding might lead to cytoprotective G_i signaling even with normal thrombin cleavage¹¹³. Interestingly, there is some indication that occupation of EPCR by either aPC or zyPC is equally able to induce these cytoprotective responses^{113, 119}, which given majority occupation of EPCR with PC suggests the default pathway is cytoprotective¹¹³.

Regardless of the specific mechanism, in this context, aPC and/or thrombin cleavage of PAR-1 on the endothelium leads to G_i intracellular signaling that can alter gene expression in endothelial cells, resulting in anti-inflammatory activities, protection from apoptosis, and tightening of the endothelial cell-cell junctions.¹⁰⁹ Modulation of gene expression includes inducing expression of various anti-inflammatory cytokines and receptors while downregulating nuclear transcription factor κ B (NF κ B) and thus various adhesion molecules, inflammatory cytokines, and apoptotic factors^{109, 120, 121, 122, 123, 124}. Additionally, PAR-1 signaling pathway results in downstream activation of sphingosine kinase-1 (SphK-1) followed by formation of sphingosine-1-phosphate (S1P) and signaling through the S1P receptor-1 (S1P₁),^{109, 125} which mediates aPC's barrier protective effects by stabilizing the endothelial cell cytoskeleton and reducing vascular permeability^{109, 126, 127}.

With two distinct activities of aPC, the question then becomes how is aPC controlling cancer progression? With the extensive evidence that coagulation enhances metastasis and anti-coagulants inhibit tumor formation, there seems to be a place for the anti-coagulative properties of aPC in cancer modulation. However, a study utilizing anti-aPC antibodies demonstrated that knocking out aPC function completely—*i.e.* both the cytoprotective and anti-coagulation effects—enhances tumor formation in a model of metastasis, but just blocking anti-coagulation while leaving cytoprotection intact does not. Furthermore, stimulating S1P₁ with an agonist can reverse the enhanced metastasis caused by the complete anti-aPC blocking antibody.¹⁰³ Together, these findings suggest that endogenous aPC limits tumor metastasis through aPC's cytoprotective activities via PAR-1 and downstream S1P₁ signaling. An additional study has also shown that overexpression of EPCR in transgenic mice resulted in reduced tumor formation in the same murine model of metastasis,¹⁰⁴ implicating involvement of EPCR. While compelling, these studies do not resolve whether exogenous aPC-mediated protection works through the same cytoprotective mechanism or how, exactly, EPCR is involved.

IV. Dissertation goals

The goals of this dissertation are two-fold:

- (1) While tolerization to the transgene following AAV-based expression in the liver has been shown to induce tolerance and prevent inhibitor formation in canine and murine models of hemophilia, the ability of such gene therapy to reverse an ongoing immune

response in an outbred, large-animal model is yet unknown. In Chapters 2 and 3, I investigate the ability of liver-directed, AAV gene therapy to eradicate anti-factor, inhibitor-based immune responses in outbred, large-animal canine models of hemophilia A and B, and I begin to explore the possible mechanisms through which this immune modulation takes place. Chapter 2 focuses on hemophilia A and attempts to characterize the induced CD4⁺ CD25⁺FoxP3⁺ T cell (potential Treg) response. Chapter 3 concentrates on hemophilia B and the cytokine profile of a dog undergoing inhibitor eradication following gene therapy. I hypothesized that AAV mediated liver expression would successfully eradicate anti-FVIII and anti-FIX inhibitors in outbred hemophilia A and B dogs through a mechanism involving induced Tregs.

(2) Activated protein C has been shown to have anti-metastatic effects, with published data suggesting the endogenous aPC's cytoprotective effect mediated through the PAR-1/S1P₁ intracellular signaling pathway is primarily responsible, with integral involvement of EPCR. Additionally, there is widespread support for the idea that modulation of the clotting cascade can also have a negative effect on tumor progression, indicating that perhaps aPC's anticoagulation functions could be involved in the ability of high doses of recombinant aPC to greatly reduce the number of metastases seen in a murine model of metastatic melanoma. In Chapter 4 I thoroughly examine the necessity and sufficiency of aPC's anticoagulant properties and cytoprotective effects in its ability to protect against tumor metastasis. In Chapter 5 I further examine the possible mechanism(s) of action of aPC- and zyPC-mediated protection against metastasis, focusing on EPCR and other

previously identified putative white blood cell and platelet receptors ¹¹³. I hypothesized that aPC and zyPC work through a common, novel mechanism, independent of both the anti-coagulative and cytoprotective effects of the protein C pathway.

CHAPTER 2

ERADICATION OF NEUTRALIZING ANTIBODIES TO FACTOR VIII IN CANINE HEMOPHILIA A FOLLOWING LIVER GENE THERAPY*

I. Introduction

Hemophilia A (HA) is an X-linked bleeding disorder characterized by deficiency in the activity of factor VIII (FVIII), a key component of the coagulation cascade. The disease occurs in approximately 1 in 10,000 live births worldwide, and >40% of these patients have severe disease, characterized by factor VIII activity <1% of normal.¹²⁸ Intravenous infusion of plasma-derived or recombinant FVIII is the standard treatment. Antibodies that neutralize the protein replacement therapy (clinically termed “inhibitors”) develop in 20-30% of young, severe and moderate HA patients, resulting in high morbidity and mortality^{129, 130}, and are also a growing problem for adults.^{131, 132} Risk factors for inhibitor formation include both genetic and environmental factors. The underlying mutation in the FVIII gene such as large gene deletions, nonsense mutations, and the most common mutation in severe HA patients—the inversion of intron 22—are all associated with inhibitor formation. However, it is not possible to predict with certainty which patients will develop inhibitors, and thus, preventive strategies are not currently feasible.^{133, 134, 135} Patients with high titers of inhibitors, defined as >5 Bethesda Units

*Most text and figures taken or modified from Finn JD, Ozelo MC, Sabatino DE, Franck HWG, Merricks EP, Crudele JM, Zhou S, Kazazian HH, Lillicrap D, Nichols TC, Arruda VR. Eradication of neutralizing antibodies to factor VIII in canine hemophilia A following liver gene therapy. *Blood*. 2010;116(26):5842-5848.

(BU) cannot usually be treated with FVIII replacement, necessitating the use of extremely expensive products that bypass the procoagulant effect of FVIII.¹²⁸ Thus, strategies for eradication of inhibitors are of fundamental clinical relevance.

Currently, the only proven therapy for inhibitor eradication is based on antigen-specific immune tolerance induction (ITI) protocols that stem from observations in the 1970's that continuous administration of large amounts of FVIII protein could lead to a reduction in inhibitor titers.¹³⁶ Current ITI involves daily infusions of FVIII protein for an average of 33 months to achieve complete eradication and is commonly followed by long-term prophylaxis. This imposes enormous challenges for pediatric patients that often require central venous catheters that are associated with a high risk of infection and thrombosis. In addition, the economic burden of this strategy is remarkable (~US\$1 million) and prohibitive for many patients outside the developed world.¹²⁹

Adeno-associated viral (AAV) vectors are one of the most extensively studied and highly used vector platforms for gene therapy applications. The safety profile of AAV vectors in clinical studies enrolling adult and pediatric populations has been excellent.^{137, 138, 139, 140} The first clinical studies using AAV to deliver the factor IX gene to the muscle or liver in subjects with hemophilia B were safe, without sustained toxicity.^{137, 141, 142} The therapeutic doses defined in canine hemophilia B models were excellent predictors of the efficacy observed in clinical trials.^{143, 144} Thus, the use of large animal models has been

essential for the successful translation of gene therapy protocols from the bench to the clinic.¹⁴⁵

Liver-directed gene expression by AAV vectors has been associated with antigen-specific immune tolerance induction in naïve adult large animals, including severe HA dogs.^{144, 145, 146, 147, 148, 149, 150} More difficult than preventing an immune response is the challenge of reversing an ongoing immune response to FVIII. We hypothesize that continuous expression of FVIII following AAV liver gene transfer could mimic ITI protocols with the additional advantage that after inhibitor eradication, the continuous expression of FVIII above 1% of normal would convert the disease phenotype from severe to moderate or mild.

II. Materials and Methods

AAV vector production

Recombinant AAV vectors were produced by a triple transfection protocol as previously described,¹³⁷ using plasmids expressing canine FVIII (cFVIII) light chain (LC) or heavy chain (HC) in separate vectors under the control of a liver-specific promoter (human thyroxine binding globulin, TBG)¹⁴⁷ or expressing cFVIII B-domain deleted (BDD) in a single vector under the control of a liver-specific promoter/enhancer (human alpha 1 antitrypsin, hAAT), a second plasmid supplying adenovirus helper functions, and a third plasmid containing the AAV-2 rep gene and the AAV-8 cap gene. Vectors were purified by repeated cesium chloride density gradient centrifugation. Vectors were generated in the Children's Hospital of Philadelphia Research Vector Core.

Animal procedures

All animal experiments were approved by the Institutional Animal Care and Use Committee at the Children's Hospital of Philadelphia, the University of North Carolina (UNC) at Chapel Hill, and Queen's University. Four adult male HA dogs with pre-existing anti-FVIII inhibitors were administered 2.5×10^{13} vg/kg of AAV8-cFVIII-LC and 2.5×10^{13} vg/kg AAV8-cFVIII-HC intravenously via the saphenous vein in a total volume of 10 mL/kg PBS (K01, K03, L44, and Wembley). Pooled normal plasma was concurrently given for cFVIII replacement to dog K03 to control an ongoing bleeding episode from a previous jugular vein puncture the day prior to vector delivery.

Control animals for Treg responses: One HA dog without pre-existing inhibitors was administered 6.0×10^{12} vg/kg of AAV8-cFVIII-HC and AAV8-cFVIII-LC (Linus), one was administered 4×10^{13} vg/kg AAV8-HCR-hAAT-cFVIII-BDD (M06), and one was administered 2×10^{13} vg/kg of AAV8-HCR-hAAT-cFVIII-BDD (L51), all by peripheral venous injection.

Systemic and local toxicology

Hematologic and comprehensive biochemical analyses of blood and serum samples for liver and kidney function tests were performed as previously described.^{146, 150}

Canine FVIII antigen, activity and antibody assays

The whole blood clotting time (WBCT) was determined as previously described.¹⁵¹

Pooled normal canine plasma was used as a standard for the quantitation of the activity by Chromogenix Coatest SP4 FVIII (Diapharma, Lexington, MA). cFVIII-LC antigen levels were analyzed by enzyme-linked immunosorbent serologic assay (ELISA) using a monoclonal antibody against cFVIII-LC (2C4.1C3) as capture antibody as previously described.¹⁵¹ Anti-cFVIII antibodies were detected by Bethesda assay or as cFVIII-specific IgG antibodies by ELISA as previously described.¹⁵¹ It should be noted that the detection of inhibitor titers less than 1 BU is unreliable in the canine HA system.

Flow cytometry

Anti-canine CD25 antibody (P4A10) was generously provided by V.K. Abrams (Seattle, WA)¹⁵² and was conjugated to AlexaFluor 488 using a commercially available kit (Invitrogen, Carlsbad, CA). Subsequently, P4A10-eFluor 660 was purchased from eBioscience (San Diego, CA). Peripheral blood mononuclear cells (PBMCs) from K01, K03 and L44 were surface stained for canine CD4-RPE (AbD Serotech, Raleigh, NC) and CD25-AF488 (P4A10) and intracellular stained with a cross reactive mouse FoxP3 – APC (eBioscience, San Diego, CA). PBMCs from L51, Linus, and M06 were surface stained for canine CD3-FITC (AbD Serotech), CD4-RPE (AbD Serotech), and CD25-eFluor660 (eBioscience) and intracellular stained for mouse FoxP3 –PerCP-Cy5.5 (eBioscience). Samples were run on a BD Canto flow cytometer and data was analyzed using FlowJo software (Treestar, Ashland, OR).

Protein infusion for immunologic challenges and pharmacokinetic analysis

Recombinant B-domain deleted canine FVIII (rBDD-cFVIII) purified protein was infused intravenously (100 IU/kg) for pharmacokinetics assessment and blood was collected at time points indicated. Canine FVIII levels were determined by ELISA and the half-life was calculated as previously described.¹⁵¹ Immunologic challenges were carried out by infusion of 25 IU/kg/dose body weight of rBDD-cFVIII on a weekly basis (total 4 doses). Pooled normal dog plasma was infused at 25 ml/dose in a similar fashion.

III. Results

Pre-existing inhibitor dogs

We used two strains of severe HA dogs prone to inhibitor formation to test our hypothesis that continuous expression of FVIII could eradicate inhibitors. These dogs have circulating FVIII antigen and activity levels <1% of normal and faithfully reproduce many of the symptoms and phenotype of severe hemophilia in humans. Moreover, the causative mutation in both canine models^{153, 154} mimics the intron 22 inversion observed in ~40% of severe disease in humans.¹⁵⁵ The first strain is from a subset of the UNC-Chapel Hill colony that developed anti-cFVIII inhibitory antibodies upon exposure to normal canine plasma.¹⁴⁵ The second strain is from the Queen's University dog colony, which has a high risk of inhibitor formation upon cFVIII protein replacement.^{156, 157} This immunological phenotype makes these subsets of dogs more representative of the human hemophilia population, and dogs with inhibitors are ideal candidates for testing the safety and efficacy of AAV-mediated immune tolerance induction protocols.

Due to the large size of the canine F8 gene—even the fully functional B-domain deleted FVIII¹⁵¹—and the limited packaging capacity of AAV vectors (4.7 Kb), the cFVIII cDNA was divided into two different AAV8 vectors expressing either the cFVIII LC or HC under control of a liver-specific promoter.¹⁴⁷

Three HA dogs with inhibitors (K01, K03 and L44) from the UNC-Chapel Hill dog colony were administered 2.5×10^{13} vg/kg of each AAV8-cFVIII-LC and AAV8-cFVIII-HC vector (5×10^{13} vg/kg total). The clinical characteristics of these dogs are shown in **Table 2.1**. The inhibitory antibodies identified in these dogs are restricted to the IgG2 subclass (equivalent to IgG4 in humans,¹⁵⁸ the most common inhibitor subclass).

To overcome the challenges of achieving hemostasis in these fragile animals, the vector was delivered by peripheral intravascular administration via saphenous vein. Thus, no exogenous recombinant cFVIII or transfusion of normal plasma was required during the vector infusion (except in K03, see subsequent pages).

Table 2.1. Summary of inhibitor eradication in HA dogs following cFVIII expression by AAV vector

Dog	Age, yr	Body weight, kg	Inhibitors			cFVIII activity plateau	Bleeds per month	
			Duration before treatment	Historical, BU	Time to eradication, wk		Pre	Post
K01	1.7	20.1	8 mo	12	5	1.5 %	3/20	2*/80
K03	1	19.3	7 mo	12	4	8 %	7/12	2*/85
L44	0.7	16	4 mo	4.5	4	1.5 %	5/8	4†/75
Wembley	4.9	16.5	~2 yr	3.6	100	1.8 %	-	-
Total							15/40	8/235

*One bleed associated with liver biopsy.

†One bleed associated with trauma from a dogfight.

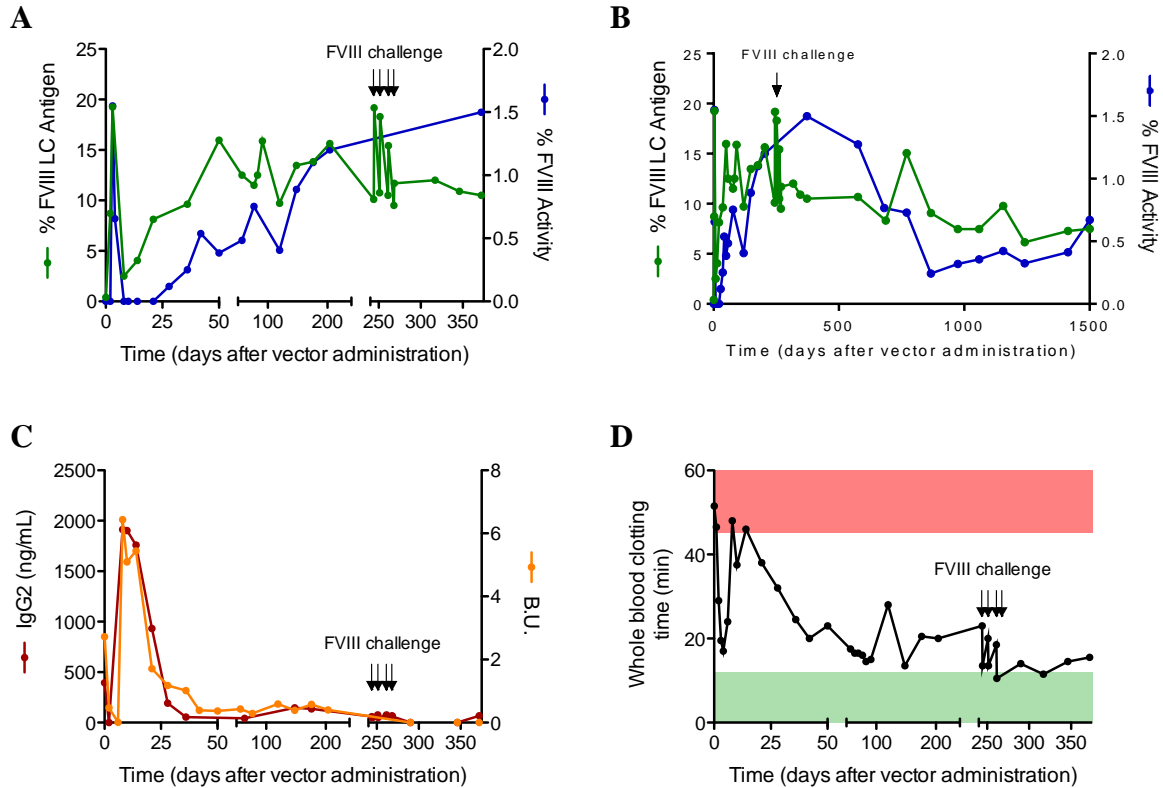


Figure 2.1 cFVIII expression and anti-cFVIII antibody responses in Chapel Hill hemophilia A dog K01 following liver delivery of AAV-cFVIII. An HA dog (K01) with pre-existing inhibitors to cFVIII was administered 2.5×10^{13} vg/kg of AAV8-TBG-cFVIII-HC and AAV8-TBG-cFVIII-LC by peripheral venous injection. (A,B) cFVIII antigen levels were assayed by a cFVIII-LC specific ELISA and activity was monitored by Coatest. (C) Anti-cFVIII antibody responses were measured by anti-cFVIII IgG2 ELISA and Bethesda assay. (D) WBCT for a normal dog is shown in green (<12 minutes), and WBCT for an HA dog is down in pink (>45 minutes). Black arrows indicate 4 weekly challenges with 500 U recombinant B-domain deleted cFVIII.

K01 had a historical maximum inhibitor titer of 12 BU and his inhibitor titer at the time of treatment was ~3 BU. After vector administration we observed a rapid increase in cFVIII expression (**Figure 2.1A**), peaking at day 3 (38 ng/mL LC antigen, 1.5% activity). This is consistent with the pattern of early expression of AAV-8 vectors. However,

transgene expression levels decreased to near background levels for 3-4 weeks and then slowly increased over time to reach cFVIII plateau levels of 30 ng/ml LC antigen and 1.5% activity. Inhibitor titers followed an inverse relationship with cFVIII antigen and activity levels. There was an initial decrease in inhibitor titer to undetectable levels, followed by rapid increase, peaking at 6 BU on day 8 and then slowly decreasing over time, and no longer detectable by day 42 (**Figure 2.1C**). During this time, we documented an increase in inhibitor titers corresponding to a decrease in cFVIII expression, indicating an anamnestic inhibitor response followed by inhibitor eradication. This is a common observation during the early phase of ITI.¹⁵⁹ K01 has been followed for more than four years with sustained cFVIII expression (**Figure 2.1B**).

The kinetics of cFVIII expression in dogs L44 (2.2 BU) and K03 (3 BU) were similar to K01. It should be noted that K03 received a transfusion of normal canine plasma at the time of vector injection to control bleeding from a jugular puncture received the previous day when collecting baseline samples. Thus the cFVIII antigen and activity from the transfusion confounds the quantification of the AAV-cFVIII mediated expression at early time points (days 2-3). In both dogs there was a rapid increase in the circulating cFVIII levels followed by a decrease to undetectable levels from 7 to 21 days (**Figure 2.2A, 2.3A**). This transient decrease in transgene expression is not observed in non-inhibitor hemophilia dogs administered AAV and is an indication of an anamnestic immune response against cFVIII.^{144, 146, 149, 150} Inhibitor titers increased starting 1 week after treatment followed by a slow decrease with complete eradication 4-5 weeks post AAV

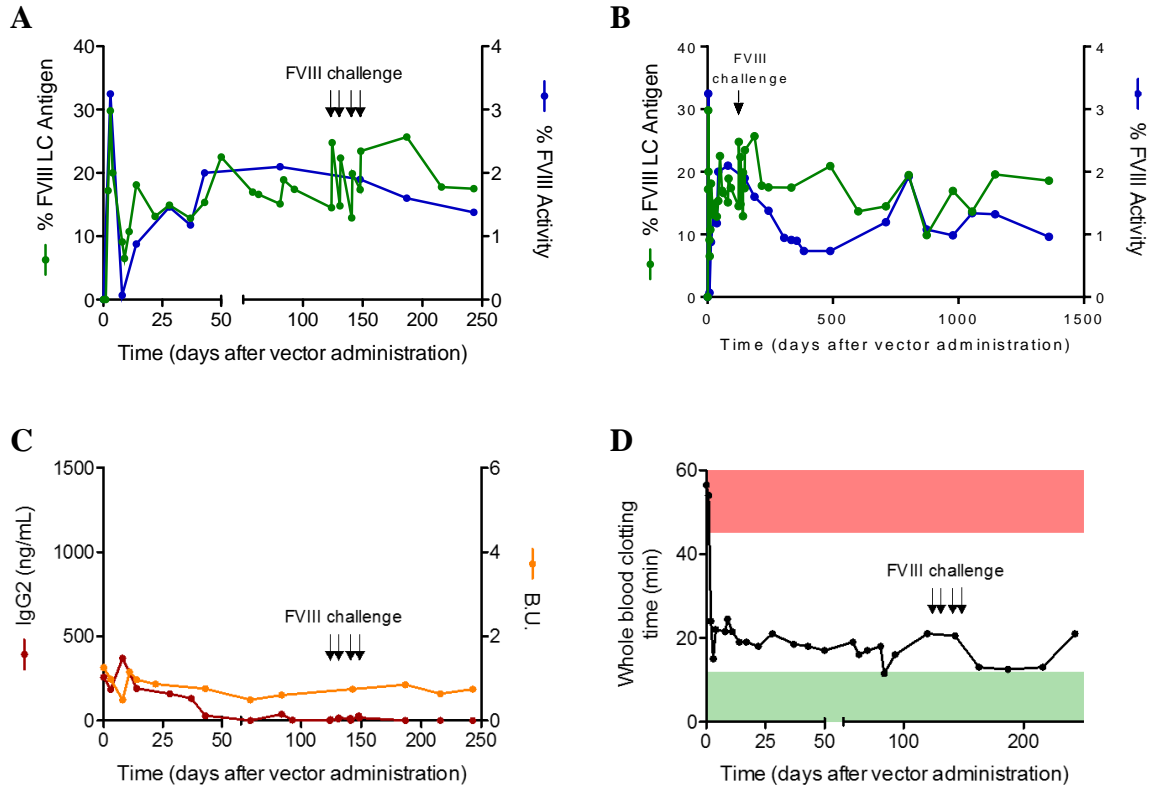


Figure 2.2 cFVIII expression and anti-cFVIII antibody responses in Chapel Hill hemophilia A dog L44 following liver delivery of AAV-cFVIII. An HA dog (L44) with pre-existing inhibitors to cFVIII was administered 2.5×10^{13} vg/kg of AAV8-TBG-cFVIII-HC and AAV8-TBG-cFVIII-LC by peripheral venous injection. (A,B) cFVIII antigen levels were assayed by a cFVIII-LC specific ELISA and activity was monitored by Coatest. (C) Anti-cFVIII antibody responses were measured by anti-cFVIII IgG2 ELISA and Bethesda assay. (D) WBCT for a normal dog is shown in green (<12 minutes), and WBCT for an HA dog is down in pink (>45 minutes). Black arrows indicate 4 weekly challenges with 500 U recombinant B-domain deleted cFVIII.

delivery (**Figure 2.2C, 2.3C**). L44 and K03 have been followed for more than four years with sustained cFVIII expression (**Figure 2.2B, 2.3B**).

We observed an expected inverse relationship between cFVIII expression and inhibitor

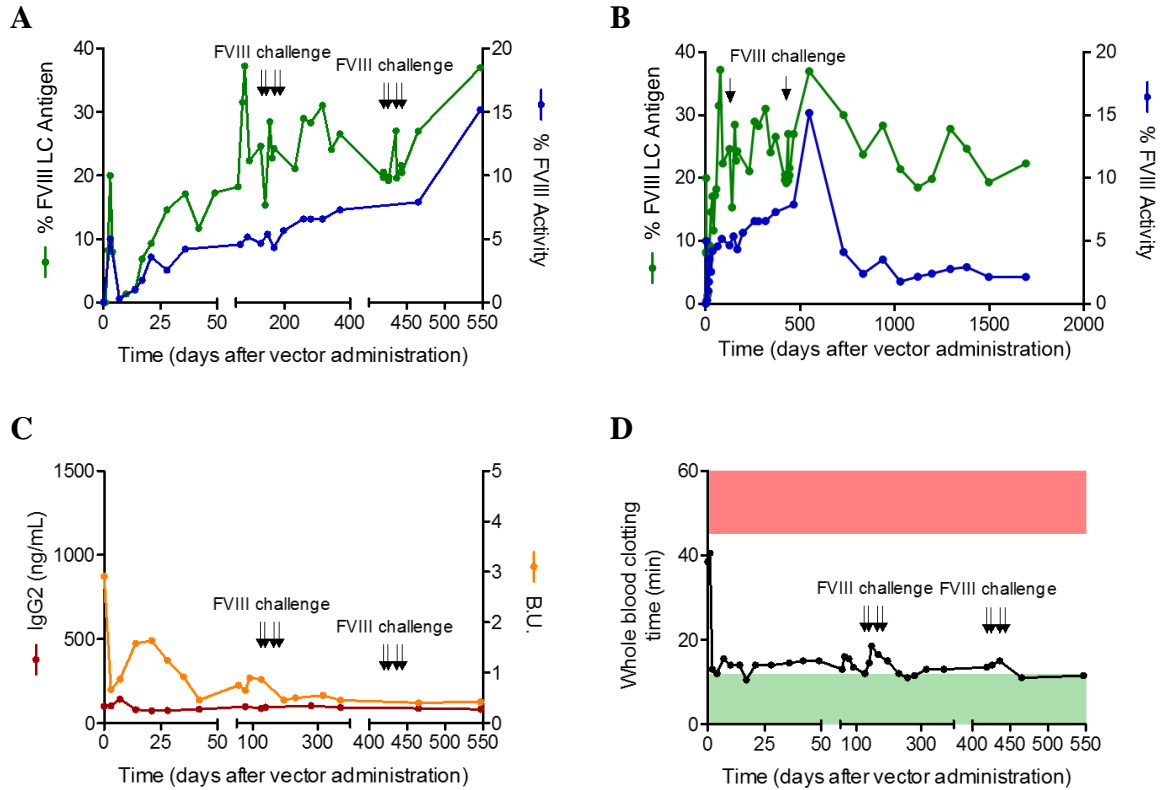


Figure 2.3 cFVIII expression and anti-cFVIII antibody responses in Chapel Hill hemophilia A dog K03 following liver delivery of AAV-cFVIII. An HA dog (K03) with pre-existing inhibitors to cFVIII was administered 2.5×10^{13} vg/kg of AAV8-TBG-cFVIII-HC and AAV8-TBG-cFVIII-LC by peripheral venous injection. (A,B) cFVIII antigen levels were assayed by a cFVIII-LC specific ELISA and activity was monitored by Coatest. (C) Anti-cFVIII antibody responses were measured by anti-cFVIII IgG2 ELISA and Bethesda assay. (D) WBCT for a normal dog is shown in green (<12 minutes), and WBCT for an HA dog is down in pink (>45 minutes). Black arrows indicate 4 weekly challenges with 500 U recombinant B-domain deleted cFVIII (starting day 140) and 4 weekly challenges with 25 ml of pooled normal canine plasma (starting day 400).

titers, with cFVIII levels steadily increasing as inhibitor titers decreased to undetectable levels. cFVIII levels stabilized at 1-2% (for dogs L44 and K01) and 8% for K03. The reasons for this discrepancy in cFVIII expression levels are unclear; however, we

previously showed that normal hemostasis at the time of AAV2 vector delivery enhances transgene expression in murine models.¹⁶⁰ Thus, it is possible that correction of hemostasis by normal plasma infusion in K03 may have contributed to the higher efficiency of gene transfer.

A consequence of using a dual-chain approach is an imbalance in circulating cFVIII LC and HC antigen levels, with the LC antigen being secreted 10-25 times more efficiently than the HC.^{161, 162} A large proportion of cells are presumably transduced with only one of the vectors, and these cells will produce cFVIII antigen that is inactive without its complementary chain. Thus, cFVIII antigen levels are higher than the cFVIII activity levels (~10 fold). We speculate that this excess of antigen is perhaps beneficial in inducing immune tolerance by increasing the overall amount of circulating antigen, as shown before in murine models.¹⁶³ The cFVIII activity observed in these dogs reached therapeutic levels as demonstrated by a sustained shortening of the whole blood clotting time (**Figure 2.1D, 2.2D, 2.3D**) in all three dogs and a remarkable improvement of the disease phenotype with reduction of more than 90% of bleeding episodes (**Table 2.1**).

In order to determine whether these animals were tolerant to cFVIII, we performed immunological challenges with purified recombinant B domain-deleted cFVIII (rBDD-cFVIII).¹⁵¹ K01, K03, and L44 were challenged with 4 weekly intravenous injections of 25 IU/kg body weight (2.5 µg/kg) of rBDD-cFVIII initiated on various days post vector

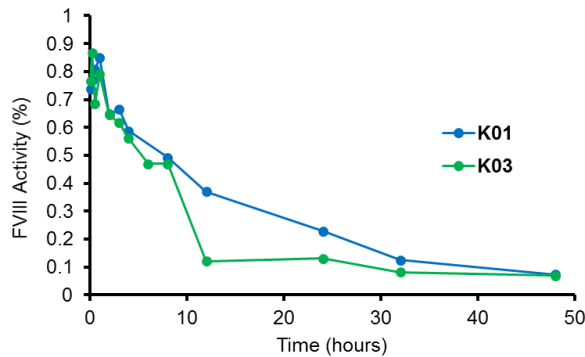


Figure 2.4 Recovery of cFVIII following intravenous administration. Recombinant B-domain deleted (rBDD) cFVIII was administered (100 IU/kg) to K01 and K03 by intravenous injection and cFVIII activity was monitored over time by Coatest assay.

administration (day 240, 140, and 113 respectively) and monitored for inhibitor formation. As can be seen in **Figures 2.1-2.3**, there was no change in cFVIII expression levels or indication of either inhibitor formation or non-neutralizing antibodies after challenge in any dog. In order to confirm that tolerance induction is sustained upon exposure to the wild type (full-length) cFVIII, we further challenged K03 starting on day 400 with 4 weekly injections of 25 ml of pooled normal canine plasma per dose. Once again, no evidence of inhibitors or antibodies to cFVIII was observed (**Figure 2.3**).

In order to further confirm the eradication of inhibitors and exclude the presence of non-neutralizing antibodies that might increase the clearance of cFVIII, we determined the recovery and half-life of cFVIII protein in K01 and K03. Both dogs were infused with 100 IU/kg of rBDD-cFVIII and plasma was collected 5 minutes to 48 hours post-infusion. As can be seen in **Figure 2.4**, there was an excellent recovery of more than 80% of the infused protein measured at 5-10 min post injection. We determined similar cFVIII fall-off curves, with a terminal half-life of ~14hrs in both dogs. These findings are comparable to our previously reported data on pharmacokinetic parameters obtain in naïve HA dogs.¹⁵¹

As previous data using liver directed gene transfer to prevent immune responses in animal models has shown the involvement of regulatory T cells,^{164, 165, 166, 167} we used flow cytometry to determine the frequency of CD4⁺CD25⁺FoxP3⁺ T cells at baseline and various subsequent time points over the first 150 days after vector administration. Interestingly, we observe an increase in CD4⁺CD25⁺FoxP3⁺ T cells at weeks 1 and 2, with a return to baseline levels by week 4 (**Figure 2.5A, Table 2.2**). When the CD4⁺CD25⁺FoxP3⁺ T cell kinetics of these dogs were compared with two non-inhibitor HA dogs (M06 and Linus) from UNC treated with the same or similar AAV vectors, the same expansion was not seen (**Figure 2.5A**). M06 and Linus had never had nor ever developed inhibitors. Conversely, L51 was an HA dog from UNC's inhibitor-prone sub-colony with no history of inhibitors upon AAV administration that developed a transient inhibitor after treatment (**Figure 2.5B**). The frequency of L51's CD4⁺CD25⁺ FoxP3⁺ T cells plummeted during inhibitor onset, but then rose concurrently with the eventual induction of FVIII tolerance. The kinetics of CD4⁺CD25⁺FoxP3⁺ T cell frequencies in these dogs suggests that the expansion of CD4⁺CD25⁺FoxP3⁺ T cells seen in K01, K03 and L44 is not simply a response to the influx of AAV vector or onset of FVIII expression. Rather, the data is suggestive of an expansion of tolerance-inducing Tregs as has been seen in previous AAV liver-directed murine and non-human primate studies.^{164, 165, 166} While further studies are required to determine the exact mechanism of tolerance induction in this model, our data is consistent with the hypothesis that regulatory T cells might be, at least in part, involved in this phenomenon.

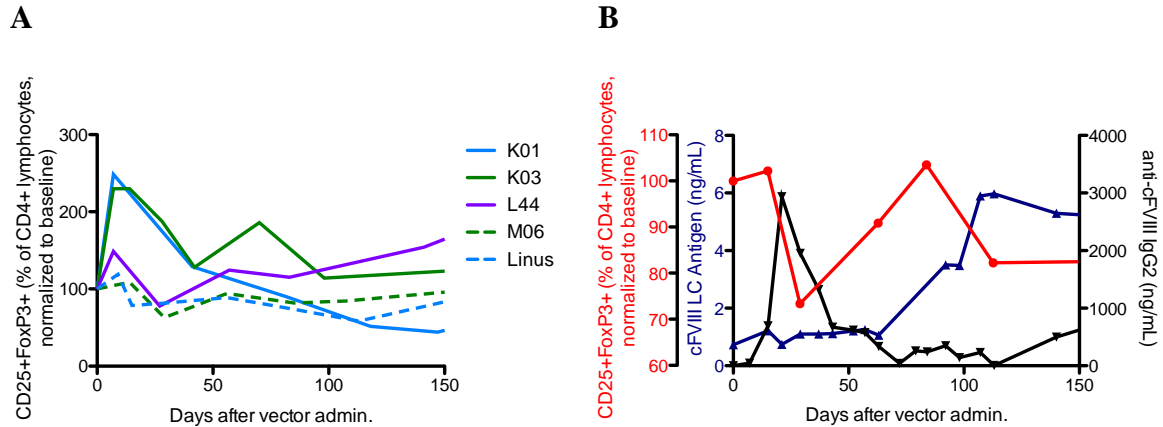


Figure 2.5 Frequency of CD4⁺CD25⁺FoxP3⁺ T cells following liver delivery of AAV-cFVIII in Chapel Hill hemophilia A dogs with and without inhibitors. (A) HA dogs with pre-existing inhibitors to cFVIII (K01, K03, L44) were administered 2.5×10^{13} vg/kg of AAV8-TBG-cFVIII-HC and AAV8-TBG-cFVIII-LC by peripheral venous injection. HA dogs without pre-existing inhibitors were administered 6.0×10^{12} vg/kg of AAV8-TBG-cFVIII-HC and AAV8-TBG-cFVIII-LC (Linus) or 4×10^{13} vg/kg AAV8-HCR-hAAT-cFVIII-BDD (M06) by peripheral venous injection. PBMCs collected before and periodically after AAV administration were collected and stained for CD3, CD4, CD25, and FoxP3 and analyzed by flow cytometry. Cells were gated by size on the lymphocyte population and the frequency of CD25⁺FoxP3⁺ cells in the CD3⁺CD4⁺ T cell population was quantitated and normalized to baseline. (B) An HA dog (L51) from the inhibitor prone population that had no pre-existing inhibitor to cFVIII was administered 2×10^{13} vg/kg of AAV8-HCR-hAAT-cFVIII-BDD by peripheral venous injection. PBMCs were collected before and periodically after AAV administration and stained and analyzed as described in A (red line). cFVIII antigen levels (blue line) were assayed by a cFVIII-LC specific ELISA and anti-cFVIII antibody responses (black line) were measured by anti-cFVIII IgG2 ELISA.

Table 2.2 Flow cytometry analysis of total PBMC's from Chapel Hill inhibitor dogs

Dog	% CD25 ⁺ FoxP3 ⁺ of total CD4 ⁺ T cells (SD*)		
	Baseline (d0)	Peak (w1-2)	Plateau (w12)
K01	1.05 (0.09)	2.61 (0.30)	0.94 (0.06)
K03	2.45 (0.05)	4.60 (0.26)	2.58 (0.03)
L44	1.01 (0.10)	1.50 (0.11)	1.16 (0.10)

*SD = standard deviation of triplicate analysis of sample

Next we treated a dog (Wembley) from the colony at Queen's University¹⁶⁸. Wembley developed inhibitors after primary exposure to recombinant human FVIII, and these inhibitory antibodies were found to cross-react with canine FVIII. He received further infusions of cryoprecipitate containing large amounts of canine FVIII. At the time of vector injection, the inhibitor titers against cFVIII were 3.5 BU. After administration of AAV8-cFVIII vector we observed a rapid increase in cFVIII antigen and activity reaching levels of 74 ng/ml and 10%, respectively (**Figure 2.6A**). These levels quickly decreased to pretreatment levels coinciding with a remarkable increase in inhibitor titers and anti-cFVIII IgG2 (**Figure 2.6B**). A similar finding was observed for anti-cFVIII IgG1 (data not shown). His inhibitors showed a strong anamnestic response, with Bethesda titers peaking at 216 BU on day 21 (**Figure 2.6B**). We continued to monitor the levels of cFVIII and anti-cFVIII antibodies. Over the following two years the anti-cFVIII inhibitor titers gradually decreased undetectable levels. As the inhibitor titers decreased, we began to observe a rise in cFVIII LC antigen and activity levels, to 28 ng/mL and 1.8%, respectively (**Figure 2.6A**). Wembley is now 74 months out from vector administration, with stable cFVIII expression levels for 26 months. He was recently challenged with rBDD-cFVIII and will be monitored for any potential return of inhibitors. This dog resembles to a certain extent the kinetics of high responder patients with substantial increase in the inhibitor levels upon exposure to FVIII protein.¹⁵⁹ In the high responder patients, ITI failure rates increase to more than double that of non-high responders.¹²⁹ Moreover, anamnestic response with BU > 200 IU is also associated with poor response to ITI. Interestingly, Wembley's anti-human FVIII inhibitors rose from a

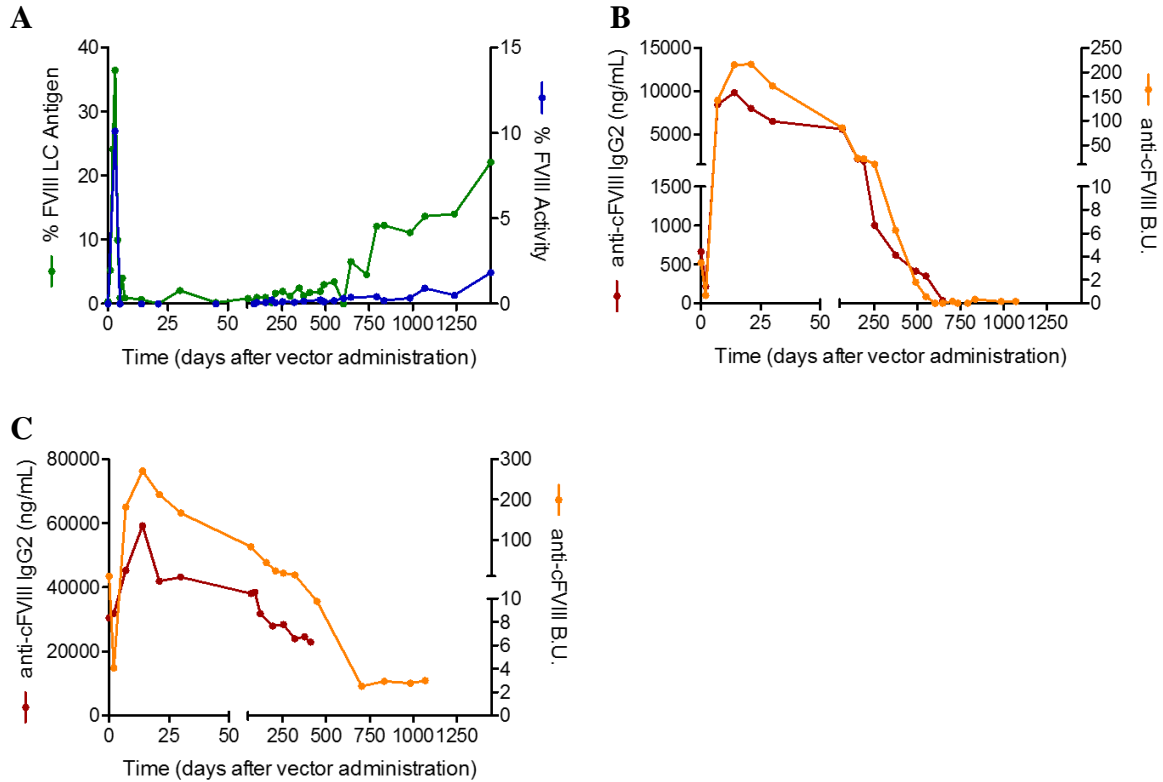


Figure 2.6 cFVIII expression and anti-cFVIII antibody responses in Queen's University high-responding inhibitor hemophilia A dog Wembley following liver delivery of AAV-cFVIII. An HA dog (Wembley) with pre-existing inhibitors to hFVIII and cFVIII was administered 2.5×10^{13} vg/kg of AAV8-TBG-cFVIII-HC and AAV8-TBG-cFVIII-LC by peripheral venous injection. (A) cFVIII antigen levels were assayed by a cFVIII-LC specific ELISA and activity was monitored by Coatest. (B) Anti-cFVIII antibody responses were measured by anti-cFVIII IgG2 ELISA and Bethesda assay. (C) Anti-hFVIII antibody responses were measured by anti-hFVIII IgG2 ELISA and Bethesda assay.

baseline value of 7.4 BU to a peak of 271 BU at d14 and have stabilized at 2.2 BU

(Figure 2.6C). A similar pattern was observed for the anti-hFVIII IgG2 levels, with current levels ~20 µg/ml. We speculate that this indicates Wembley was tolerized to

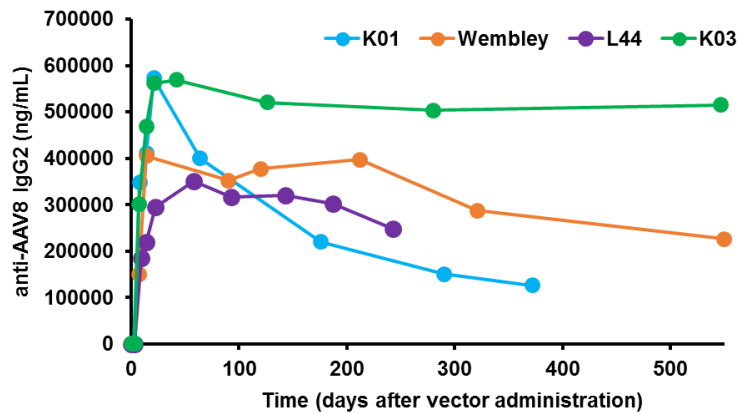


Figure 2.7 Anti-AAV8 capsid humoral responses. Anti-AAV8 capsid IgG2 responses were assayed in all four dogs. Plasma samples were assayed for capsid specific IgG2 by ELISA with plates coated with empty AAV8 capsid.

specific epitopes shared between human and canine FVIII; however, there continue to be inhibitors specific for epitopes unique to hFVIII. In order to confirm that the immune tolerance to cFVIII was specific, we measured anti-AAV8 IgG2 antibody levels. As seen in **Figure 2.7**, all animals developed a robust and sustained anti-capsid immune response, indicating that these animals are fully capable of generating and maintaining humoral immune responses to other antigens following vector administration.

IV. Discussion

The contrast in the immunological profile and response following AAV mediated expression of cFVIII between the three Chapel Hill dogs and the dog from the Queen's University colony is remarkable. Despite the fact that these dogs have a similar underlying causative mutation, all three Chapel Hill dogs (K01, K03, L44) showed mild anamnestic responses and rapid eradication of inhibitors in 4-5 weeks, while Wembley had a very strong immune response that took over a year and a half for inhibitor titers to decrease to background levels. There are several factors that may explain these distinct outcomes, including the previous exposure to xenoantigen (human FVIII) that could

hamper the ability to induce antigen-specific immune tolerance. Second, there are the differences in strains of dogs that may reflect inherited factors similar to ethnicity as a genetic risk factor in humans.^{134 135} Lastly, the long duration (~2 years) between inhibitor development and AAV administration may also influence the rates of success as observed in humans on ITI.¹²⁹

Collectively, the data presented here demonstrate for the first time the potential of liver-directed, AAV-mediated gene expression to induce tolerance to the transgene in the setting of pre-existing inhibitory antibodies in an adult, large-animal model of disease. The sustained expression of cFVIII from the transgene after inhibitor eradication recapitulates secondary prophylactic replacement protocols required to maintain immune tolerance in patients post successful inhibitor eradication.¹²⁹ In this model, we observed both inhibitor eradication and complete normalization of pharmacokinetics of FVIII protein infusion as well as improved disease phenotype. Overall, vector administration was well tolerated with no abnormalities on serial determinations of hematologic and biochemical analyses of blood and serum samples for liver and kidney function tests.

The underlying mechanism of the success of ITI in humans is still unclear, but it has been investigated in preclinical studies and revealed to depend on both B and T cell response.^{169, 170} The exact mechanism of the immune tolerance induction in this HA dog study is currently unknown. Previous work in murine and non-human primate models has shown that sustained AAV-mediated expression of transgenes can induce tolerance, and that this

sustained expression is dependent on regulatory T cells.^{164, 165, 166} In addition, recent work using microRNA to restrict transgene expression from a lentiviral vector to hepatocytes has also shown sustained transgene expression and the induction of antigen-specific T regulatory cells.¹⁶⁷ While our observation that CD4⁺CD25⁺FoxP3⁺ T cells are transiently up-regulated following gene transfer is consistent with the hypothesis that regulatory T cells are involved, much work still needs to be done—including testing the function and antigen specificity of these cells—to fully investigate the mechanism of immune tolerance induction in this model. It is also likely that this immune tolerance induction involves multiple mechanisms including anergy and/or deletion. This is an area of research currently under investigation.

Our group and others have previously demonstrated that using viral vectors to direct gene transfer to the liver of adult^{144, 146, 148, 150} or neonatal^{171, 172} large animal models can induce tolerance to the expressed transgene and prevent immune responses. Data on tolerance induction by gene therapy and/or immune modulatory strategies in the pre-existing immune responses has been limited to murine models.^{173, 174, 175} Considering the limited numbers of HA dogs in this study and the modest inhibitor titers at the time of vector administration, these findings have to be considered a proof-of-principle that immune tolerance induction is feasible in the setting of pre-existing immunity in a large animal model for an unmet medical need. These data may have relevance not only for hemophilia but also for a variety of diseases whereby antibody formation to the therapeutic protein or enzymes could prevent optimal clinical responses.^{176, 177}

CHAPTER 3

AAV LIVER EXPRESSION OF FIX-PADUA RESULTS IN FIX INHIBITOR PREVENTION AND ERADICATION IN HEMOPHILIA B DOGS AND MICE[†]

I. Introduction

Hemophilia B (HB) is an X-linked inherited bleeding disease characterized by deficiency of factor IX (FIX) due to *F9* gene mutations. Patients with severe HB (residual FIX activity <1% normal) have recurrent bleeding episodes associated with increase morbidity and mortality compared to those with moderate (FIX 1-5%) or mild disease (FIX >5-30%). Treatment of HB is based on protein replacement therapy, and prophylactic therapy is associated with clinically beneficial outcomes. One of the main complications of protein replacement therapy is the development of antibodies (clinically termed inhibitors) to the infused protein, which occurs in 1-3% of HB patients.^{178, 179, 180} Most of these patients have severe disease, and one of the main determinants of inhibitor formation is the underlying *F9* mutation. HB patients with mutations such as missense mutations that lead to circulating but defective FIX antigen, termed cross reacting material (CRM)-positive, exhibit a lower risk of inhibitor formation compared to CRM-negative patients.^{178, 179, 181} In contrast, approximately 50% of patients with large gene deletions or rearrangements develop FIX inhibitors, followed by patients with premature

[†]Most text and figures taken or modified from Crudele JM, Finn JD, Siner JL, Martin NB, Niemeyer GP, Zhou S, Mingozi F, High KA, Lothrop Jr CD, Arruda VR. AAV liver expression of FIX-Padua results in FIX inhibitor prevention and eradication in hemophilia B dogs and mice. *Submitted to Blood*.

stop codon, frame shift or splice-site mutations (20-30%).¹⁸¹ Thus, null mutations in *F9* significantly increase the risk of inhibitor formation.

Several treatment strategies for hemophilia based on protein, nucleic acid or cell therapies are under development aimed at increasing factor levels to the range of moderate or mild disease.^{182, 183, 184, 185, 186, 187} Gene therapy using adeno-associated viral (AAV) vectors for liver FIX gene transfer is emerging as a successful strategy as long-term expression of circulating FIX and improvement of disease phenotype have been reported.^{183, 188} Data from early-phase AAV-FIX clinical trials demonstrate that immune responses to vector capsid proteins is a main safety concern and is directly correlated to vector dose.^{183, 188} Thus, strategies to reduce the vector dose are highly attractive to overcome these safety concerns.

We previously reported a case of thrombophilia associated with an arginine 338 to leucine (FIX-R338L, FIX-Padua) substitution in *F9*. The mutant FIX circulates at normal antigenic levels but exhibits 8-fold increased clotting activity (776% of normal in the proband).¹⁸⁹ Thus, the use of FIX-Padua offers an alternative strategy for treating HB. Preclinical studies in severe HB dogs provide a unique opportunity to address both efficacy and safety of a novel strategy. There are two severe canine HB models available, both of which are CRM-negative. However, the underlying *F9* mutation, mRNA levels, and risk of inhibitor formation differ.^{190, 191, 192} The University of North Carolina-Chapel Hill (UNC-CH) model is due to a missense mutation, glutamic acid 379 to glycine, which

leads to normal RNA levels but probable disruption of protein folding.^{190, 191} The University of Alabama at Birmingham (UAB) model results from a frame shift mutation, premature stop codon at position 146 (null mutation) and undetectable mRNA likely due to transcript instability.¹⁹¹ Infusion of canine FIX concentrate in naïve HB dogs resulted in inhibitor formation in the UAB model but not the UNC-CH model.^{193, 194, 195, 196} Preclinical studies using these models for AAV muscle gene therapy showed that the parameters of vector dose tested (per site, per body weight), which proved safe in the UNC-CH model, resulted in inhibitor formation in the UAB dogs.^{193, 195, 196} Therefore, in the skeletal muscle-directed AAV trial only severe HB men with *F9* missense mutations were enrolled.¹⁹⁷ With liver gene therapy, both canine models showed long-term sustained expression of FIX-WT.^{193, 194, 195, 196} Thus, patients with missense and null mutations were enrolled in AAV liver trials. Overall, none of the 15 patients enrolled in these early phase studies developed FIX inhibitors.^{188, 197, 198} Thus, data on immune responses to the transgene in these canine models are likely to be predictive of human responses.

Here we sought to determine the immunogenicity of FIX-Padua following AAV8 liver gene transfer in HB dogs with a null mutation and perform comprehensive safety studies in mice. We hypothesized that if the expression of FIX-Padua is safe, these data will further enhance the potential of clinical translation of FIX-Padua to HB patients, including those with underlying *F9* null mutations.

II. Materials and Methods

Recombinant AAV vector

Production of AAV serotype 8 vectors was carried out as previously described.¹⁹⁹ The expression cassette contained canine FIX-R338L (cFIX-Padua), human FIX-R338L (hFIX-Padua) or human FIX wild-type (hFIX-WT) behind a liver-specific promoter.¹⁹⁹

Canine studies

The Institutional Animal Care and Use Committee at the Children's Hospital of Philadelphia and the UAB approved all experiments. Three adult male HB dogs received AAV at doses of 1×10^{12} or 3×10^{12} vector genomes per kilogram body weight (vg/kg) via the saphenous vein diluted in PBS over a 30-minute period.

Systemic and local toxicity

Hematologic and biochemical analyses of blood and serum samples for liver and kidney functions and pathological activation of coagulation was monitored by D-dimer and thrombin-antithrombin (TAT) complexes levels as previously described.²⁰⁰

Canine FIX antigen, activity and antibody assays

Whole blood clotting time (WBCT), cFIX antigen and activity, and thrombelastography (TEG) were assayed as previously described.^{201, 202} Neutralizing antibodies to cFIX were determined by Bethesda assay and reported as Bethesda Units (BU), in which 1 BU inhibits 50% of clotting activity of FIX in normal canine plasma. Antibodies against cFIX

were measured by enzyme-linked immunosorbent assay (ELISA) against IgG1, IgG2 and total IgG (Bethyl Laboratories, Inc, Montgomery, TX) as previously described.²⁰⁰

Immune responses to AAV8 capsid proteins

To detect antibody responses against AAV8 capsid proteins, plates were coated with AAV8 empty capsids (1 µg/ml) as previously described.²⁰⁰ Coating with serially diluted dog reference serum with known quantities of IgG1 and IgG2 (Bethyl Laboratories, Montgomery, TX) served as a standard curve.²⁰³

Immunological challenges with canine FIX-WT protein concentrate

Dogs were challenged with 0.5 mg of pooled plasma-derived, purified cFIX concentrate (Enzyme Research Laboratory, South Bend, IN) by intravenous injection at indicated time points. Plasma samples were collected at baseline and weeks 1, 2 and 4 post protein injection. Humoral responses to cFIX-WT were monitored using assays for inhibitory and non-inhibitory antibodies to cFIX as described above.²⁰⁰

Canine multiplex cytokine array

Cytokines were assayed using a Milliplex canine cytokine MAG Panel kit (EMD Millipore, Billerica, MA) 8-plex containing mIL15-MAG, cGMCSF-MAG, cIFNG-MAG, cIL2-MAG, cIL6-MAG, cIL10-MAG, cIL18-MAG, and cTNFA-MAG.

Standards, quality controls and samples were assayed in duplicate. The bead counts and fluorescence intensity of the beads was measured using a Luminex 100/200 analyzer.

Samples were measured once, and blank values were subtracted from all readings.

Standard curves were produced and sample values determined using xPONENT software.

Murine studies

Assessing immunogenicity of hFIX-Padua

Adult HB male mice on C57Bl/6 background (n=7-9 mice/group) were injected with AAV-hFIX-WT or AAV-hFIX-Padua at 4×10^{10} vg/kg. Mice then received 2 µg/mouse of hFIX-WT or hFIX-Padua via subcutaneous injection every week for four weeks starting at week 10 post-vector. Ten weeks after the last protein injection, mice received hFIX-WT or hFIX-Padua (2 µg/mouse) in complete Freund's adjuvant, CFA, (Sigma, St Louis, MO) and then in incomplete Freund's adjuvant, IFA, (Sigma, St Louis, MO) 8 weeks later. Control group consisted of adult naïve HB mice (n=4-5 mice/group) injected with hFIX-WT or hFIX-Padua protein alone in a similar fashion. We monitored FIX antigen levels, inhibitory antibodies and murine anti-hFIX total IgG at baseline, week 4 post AAV injection and 4 weeks after the last of each protein challenge (protein alone, protein in CFA and protein in IFA).

Long-term safety studies in mouse models

Adult hemostatically normal male C57Bl/6 mice received AAV encoding hFIX-Padua or hFIX-WT at five vector doses ranging from 4×10^{10} to 2×10^{12} vg/kg (n=5 mice/dose/vector) and were followed for several months. We determined hFIX expression levels by antigen and activity assays and TAT complex levels as previously

described²⁰⁴ at 2, 5 and 8 months post vector injection. D-dimer levels were monitored only at 2 and 8 months post-vector injection due to sample volume limitation.²⁰⁴ Kaplan-Meier analysis was used to determine the survival rates; the control group (n=5) received 1×10^{11} vg/kg AAV empty capsid.

Recombinant hFIX-WT and hFIX-Padua protein production

We used an expression system consisting of pLenti6.3 (ViralPower HiPerform Expression System, Invitrogen, Carlsbad, CA) by reversal transfection to generate a stable human embryonic kidney 293 cell line expressing hFIX-WT and hFIX-Padua. Protein purification consisted of ion exchange column followed by HQ-Shepharose (Applied Biosystems, Forster City, CA) column and eluted with a calcium gradient.

III. Results

Canine Studies

Sustained expression of cFIX Padua in inhibitor-prone HB dogs is safe

Two adult HB dogs (Wick and Trex) received AAV8 encoding cFIX-Padua at doses of 1×10^{12} or 3×10^{12} vg/kg, respectively, by intravenous injection (**Figure 3.1; Table 3.1**).

At both doses the resulting FIX activity levels were an average of 7-12 times higher than the antigen levels, and these activity levels put the dogs in the range of mild hemophilia.

In these dogs the WBCT (**Figure 3.1E,F**) and the TEG (**Figure 3.2; Table 3.2**)

normalized within a week following AAV delivery. This was accompanied by a

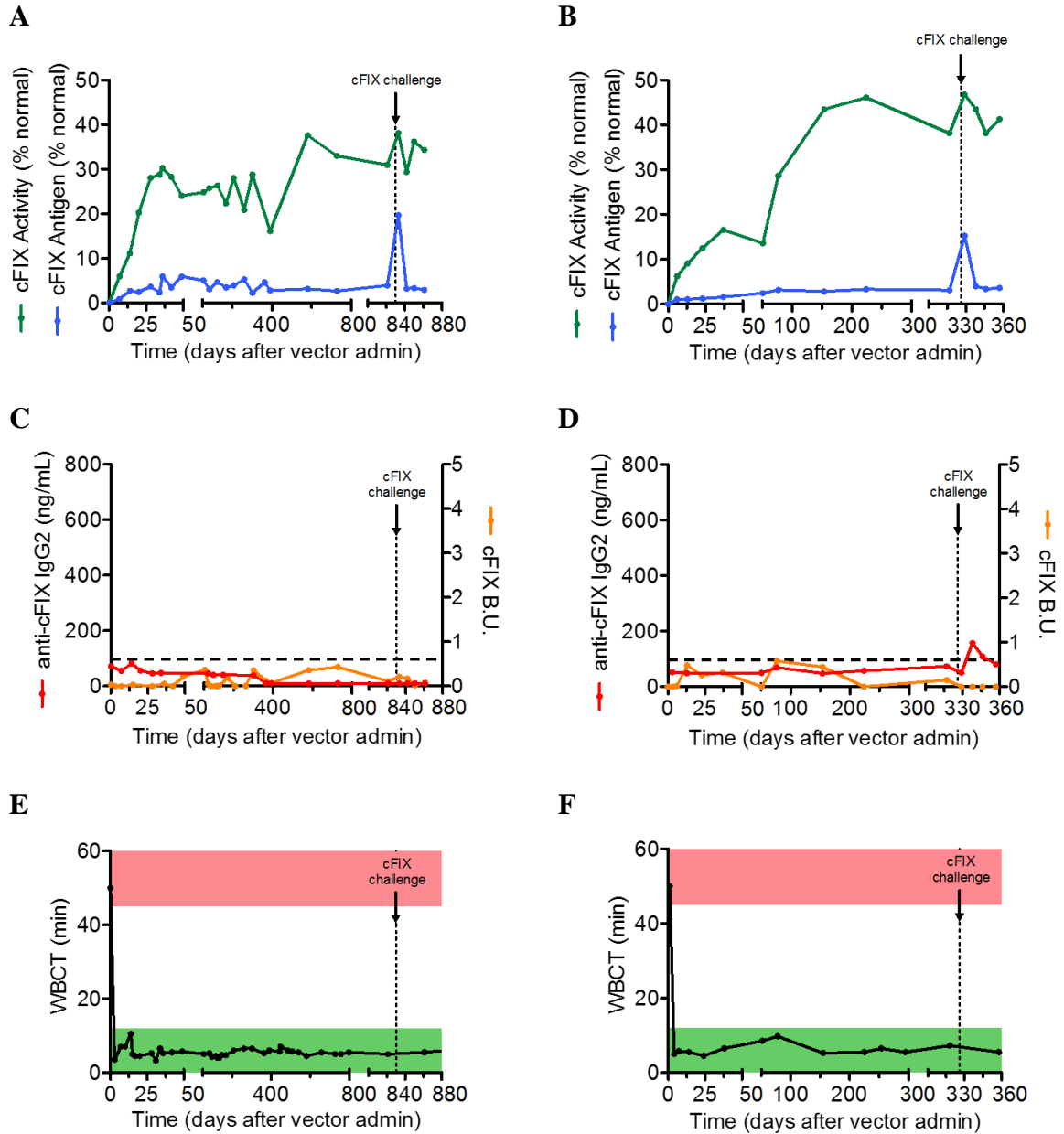


Figure 3.1 cFIX expression and anti-cFIX humoral responses in HB dogs following liver delivery of AAV-cFIX-Padua. Two University of Alabama HB dogs with no previous history of inhibitors were assessed for (A,B) cFIX antigen and activity, (C,D) anti-cFIX IgG2 and Bethesda units, and (E,F) whole blood clotting times after AAV8-cFIX-Padua administration. Trex (A,C,E) received 3 x 10¹² vg/kg and Wick (B,D,F) received 1 x 10¹² vg/kg. Horizontal dashed lines, positive levels of B.U. and IgG2; vertical dashed lines, time of challenge with recombinant cFIX; pink bar, WBCT of hemophiliac dogs (>45 min); green bar, WBCT of normal dogs (<8 min).

Table 3.1 Summary of AAV8-cFIX-Padua treatment of three adult dogs with severe HB

Dog	Age	Body weight, kg	Vector dose, vg/kg	Vector dose, total dose	cFIX-Padua plateau levels			Bleeds per month	
					Activity, % mean \pm SD	Antigen, % mean \pm SD	Specific activity	Pre-treatment	Post-treatment
Wiley	2 yr 8 mo	10.2	3×10^{12}	30.6×10^{12}	241.2 ± 31.9	28.8 ± 9.3	8.6	7/32	0/30
Trex	2 yr 8 mo	5.8	3×10^{12}	17.4×10^{12}	26.8 ± 5.5	3.81 ± 1.29	7.9	4/32	0/30
Wick	11 mo	8.6	1×10^{12}	8.6×10^{12}	39.9 ± 5.8	3.33 ± 0.38	12	0/11	0/13
Total								11/75	0/73

Table 3.2 Thromboelastography clot formation values

Dog	R, minutes	K, minutes	α , degrees	MA, mm
Wiley	5.1	1.2	71.5	70.6
Trex	4.4	1.5	68.2	67.8
Wick	6.2	2.8	54.4	59.2
WT dog	6.4	3.2	49.2	43.6
HB dog	no clot detected in 60 min assay		n/a	0.25
Normal canine range	1.8-8.6	1.3-5.7	36.9-74.6	42.9-67.9

R indicates reaction time until detection of 2 mm clot; K, clot formation time from R until clot reaches 20 mm; α , angle of line tangent to the curve at K; and MA, maximum amplitude indicating clot strength

Normal canine range from Bauer.²⁰⁵

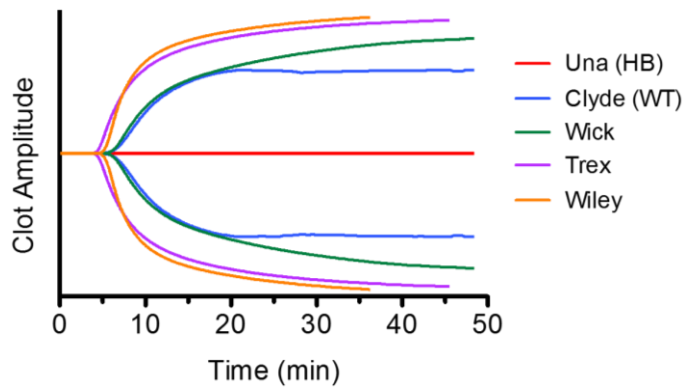


Figure 3.2 Thromboelastographs for HB dogs following liver delivery of AAV-cFIX-Padua. Thromboelastographs were run on kaolin-activated, citrated plasma collected two months after the dogs were challenged with protein. Una is an untreated HB dog; Clyde is a wild type dog.

complete lack of bleeding episodes that further support the correction of the disease phenotype (**Table 3.1**).

There was no formation of inhibitory or non-inhibitory antibodies to cFIX-Padua (**Figure 3.1C,D**) throughout the cumulative long-term follow up of ~3.5 years (ongoing observations). We specifically assay for canine IgG2 subclass, which is associated with inhibitory antibody in this HB model upon FIX protein concentrate injection or gene therapy^{199, 196}, and is the equivalent of human IgG4 (the IgG subclass associated with inhibitory antibody²⁰⁶). We also assayed for canine IgG1 (**Figure 3.3**), but found none against cFIX-Padua in either dog.

We challenged these dogs by intravenous injection of 0.5 mg cFIX-WT protein concentrate 11 months (Wick) or 2.3 years (Trex) after vector injection. We reported earlier that in this naïve HB dog strain this strategy induces the formation of inhibitory antibodies to cFIX-WT within a week post challenge¹⁹³. Here, in the AAV-cFIX-Padua

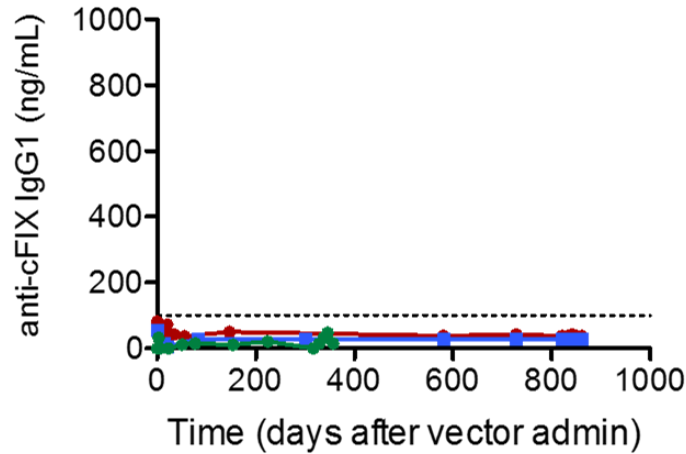


Figure 3.3 Anti-cFIX humoral responses in HB dogs following liver delivery of AAV-cFIX-Padua. In addition to the IgG2 ELISAs, the University of Alabama HB dogs were assessed for IgG1 anti-cFIX antibodies. Wiley, red; Trex, blue; Wick, green; horizontal dotted line indicates background levels of IgG1.

expressing dogs, there was no detection of antibodies to cFIX by Bethesda assay or anti-cFIX IgG2 ELISA (**Figure 3.1C,D**). Thus, expression of the cFIX-Padua variant shows increased specific activity as observed in humans with FIX-Padua¹⁸⁹ and no unwanted immune responses even upon challenge with cFIX-WT protein concentrates.

Eradication of inhibitory antibodies to FIX by liver gene therapy using AAV-cFIX-Padua

The dog Wiley developed inhibitors after primary exposure to human FIX protein, and these inhibitory antibodies cross-reacted with cFIX. At the time of vector injection, the inhibitor titers against cFIX were undetectable by Bethesda assay but anti-cFIX IgG2 levels were ~500 ng/ml. After administration of AAV-cFIX-Padua vector, we observed a

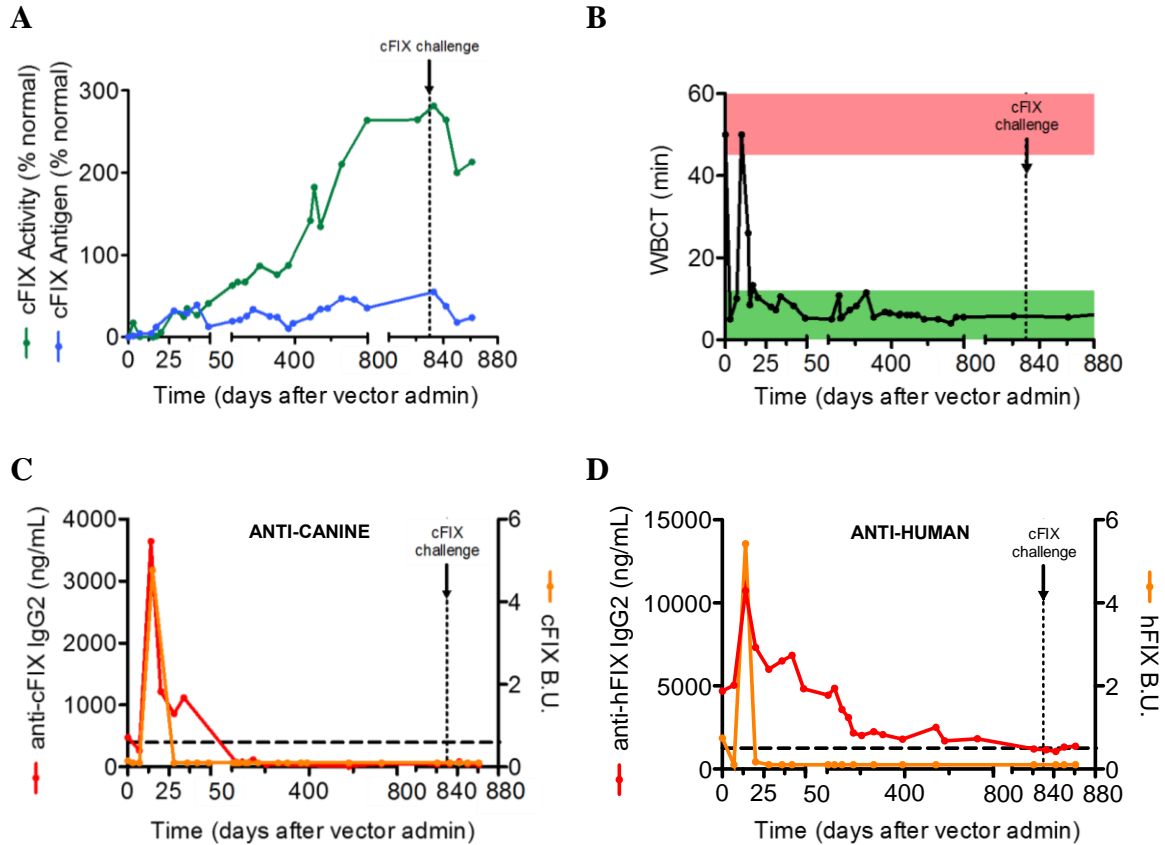


Figure 3.4 cFIX expression and anti-cFIX humoral responses in an HB dog with pre-existing anti-hFIX inhibitors after administration of AAV-cFIX-Padua. Wiley, a University of Alabama HB dog with a history of inhibitors against hFIX, was assessed for (A) cFIX antigen and activity, (B) whole blood clotting time, (C) anti-cFIX IgG2 and Bethesda units, and (D) anti-hFIX IgG2 and Bethesda units after administration of 3×10^{12} vg/kg AAV8-cFIX-Padua. Horizontal dashed lines, positive levels of B.U. and IgG2; vertical dashed lines, time of challenge with recombinant cFIX; pink bar, WBCT of hemophiliac dogs (>45 min); green bar, WBCT of normal dogs (<8 min).

rapid increase in cFIX antigen and activity, which reached levels of 2% and 20% of normal, respectively. These levels quickly decreased to pretreatment levels, coinciding with an increase in inhibitor titers and anti-cFIX IgG2 (**Figure 3.4, Table 3.1**). These

findings are consistent with the initial normalization of the WBCT by week 1 and a return to baseline levels at day 10 post vector injection. Wiley's inhibitors showed an anamnestic response, with titers peaking at ~5 BU on day 15 (**Figure 3.4C**) followed by a spontaneous decline to complete eradication by day 28. Similarly, anti-cFIX IgG2 levels peaked concomitant with the Bethesda titers but showed a slower decline, with total eradication at day 70. At no point were anti-cFIX IgG1 antibodies detected (**Figure 3.3**). We continue to monitor the levels of cFIX antigen/activity and anti-cFIX antibodies, with no evidence of cFIX antibodies observed over 2.4 years. Notably, Wiley's anti-human FIX inhibitors rose to 5.3 BU at day 14 and disappeared by day 20 (**Figure 3.4D**). The anti-hFIX IgG2 levels rose from a baseline of 5 µg/mL to a peak at day 14 of 11 µg/mL, but, although they are gradually declining, residual levels of ~1.5 µg/mL remain detectable. These data suggest that Wiley developed immune tolerance to specific epitopes shared between the human and canine FIX proteins; however, non-inhibitory antibodies specific for epitopes unique to hFIX remain. Because of the potential systemic complications associated with inhibitory antibodies to FIX such as anaphylactic reaction and nephrotic syndrome^{178, 207}, we monitored Wiley by clinical and laboratory assays. No evidence of abnormal kidney function or urinary protein loss has been observed (data not shown). To finally confirm the eradication of inhibitory antibody to cFIX and immune tolerance induction, we challenged Wiley with 0.5 mg cFIX-WT protein concentrate 2.3 years after vector injection. Again, no humoral responses were documented by Bethesda assay or anti-cFIX IgG2 ELISA (**Figure 3.4C**). Together these findings show that AAV liver expression of cFIX-Padua is efficacious in eradicating

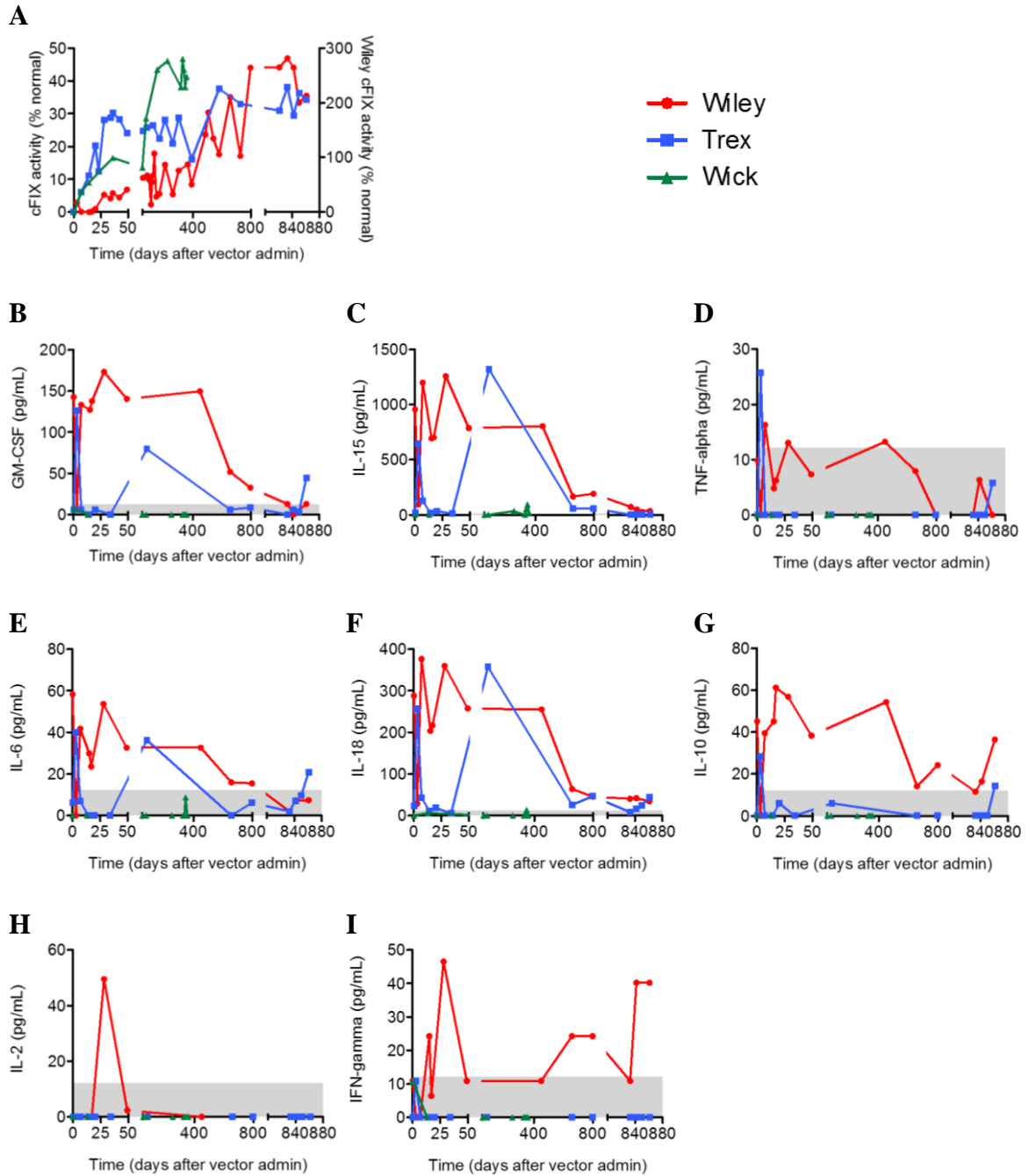


Figure 3.5 Cytokine profiles of dogs following AAV-cFIX-Padua liver gene therapy. A cytokine multiplex array was run to assess the levels of (B) GM-CSF, (C) IL-15, (D) TNF- α , (E) IL-6, (F) IL-18, (G) IL-10, (H) IL-2, and (I) IFN- γ compared to the (A) cFIX expression levels as measured by % cFIX activity in the three HB dogs (Wiley, red, right Y axis for % activity; Trex, blue; Wick, green) at baseline and following delivery of AAV. Grey bars indicate level of detection of the assay.

inhibitory antibodies to cFIX without systemic toxicity.

Cytokine profile following AAV-cFIX-Padua liver gene therapy and FIX inhibitor eradication

Cytokine profiles can be useful in tracking immune responses and suggesting immunologic mechanisms. Therefore, we ran a multiplex array on plasma from the three dogs looking at 8 cytokines, with a focus on T cell cytokines (**Figure 3.5**). Wick did not have detectable cytokine levels for any of the time points assayed. Trex showed a spike on day 3 following vector administration of CM-CSF, IL-15, TNF- α , IL-6, and IL-18, as well as small spikes of IL-10 and IFN- γ (**Figure 3.5B-G**). Interestingly, he also showed high levels of GM-CSF, IL-15, IL-6, and IL-18 on day 70 (**Figure 3.5B,C,E,F**). This did not correlate with any loss of cFIX expression or activity, detection of anti-cFIX IgG or BU, or prolongation of the WBCT (**Figure 3.1, 3.3, 3.5A**). Wiley's levels of GM-CSF, IL-15, IL-6, and IL-18 (**Figure 3.5B,C,E,F**) inversely correlated to his cFIX activity levels (**Figure 3.5A**). Conversely, he had relatively low levels of TNF- α and IL-2 throughout, with a small spike in the latter at day 27 (**Figure 3.5D,H**). Wiley's IL-10 levels climbed from weeks 2-4, generally falling thereafter until he was challenged on day 840, at which point levels began to rise again (**Figure 3.5G**). IFN- γ showed a bimodal distribution, with an early peak at week 4 followed by a late peak following the recombinant protein challenge (days 847-860) (**Figure 3.5I**).

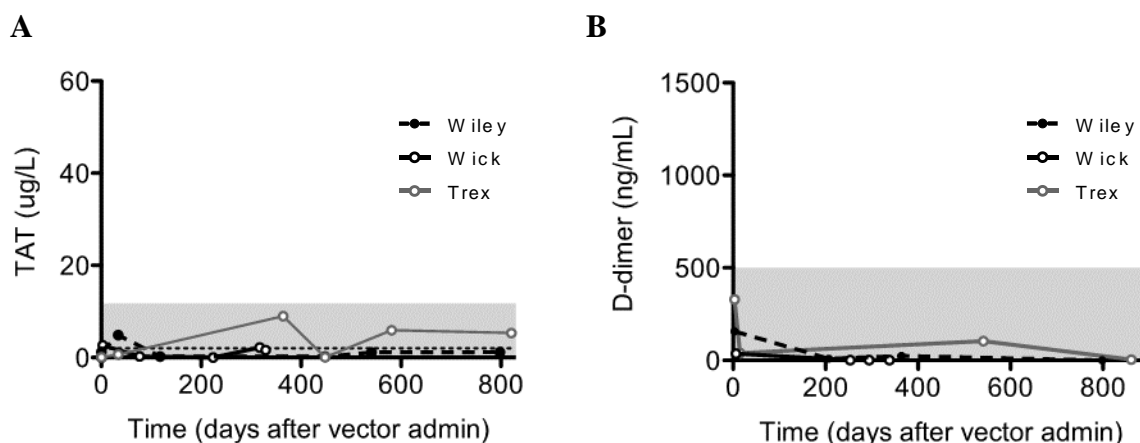


Figure 3.6 Markers of coagulation activation in HB dogs following liver delivery of AAV-cFIX-Padua. All three dogs were assessed for (A) thrombin anti-thrombin complexes (TAT) and (B) D-dimers as markers of coagulation at baseline and time points following AAV administration using ELISAs. Horizontal dotted line indicates the limit of detection of the TAT ELISA (2 ug/L). Grey bar indicates normal range (<11.7 ug/L TAT and <500 ng/mL d-dimer).

Risk of thrombogenicity of long-term expression of FIX-Padua

Expression of FIX-Padua is associated with improvement of the disease phenotype as noted by the normalization of WBCT (**Figures 3.1,3.4**), TEG parameters (**Figure 3.2; Table 3.2**), and the lack of bleeding episodes following AAV delivery (**Table 3.1**).

In both dogs expressing FIX in the range of mild hemophilia (Wick and Trex), we measured TAT complex and D-dimer levels at different time points and no abnormal

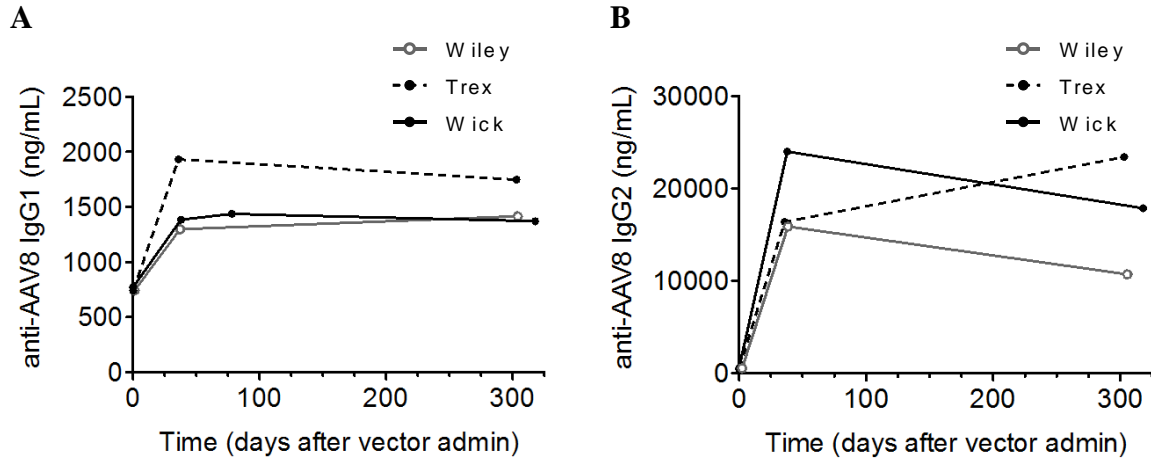


Figure 3.7 Humoral responses to AAV8 capsid in HB dogs following liver delivery of AAV-cFIX-Padua. All three dogs were assessed for anti-AAV8 (A) IgG1 and (B) IgG2 antibodies at baseline and time points following AAV administration using anti-AAV8 IgG ELISAs.

elevation in the levels were detected^{202, 208}, consistent with a lack of clinically recognized thrombosis (**Figure 3.6**). Notably, data from the dog Wiley showed continuous expression of supraphysiologic levels of FIX-Padua after inhibitor eradication for over 400 days and yet presented no clinical evidence of peripheral thrombosis. TAT and D-dimer levels remain within normal limits^{202, 208} (**Figure 3.6**). These data suggest that the risk of thrombogenicity of FIX-Padua is low at a wide range of expression levels.

Humoral responses to AAV 8 capsid

All dogs developed a robust and sustained anti-AAV8 capsid immune response measured by increase in IgG2 and, to a lesser extent, IgG1 levels (**Figure 3.7**). These data indicate that these dogs are capable of generating sustained immune responses to other antigens,

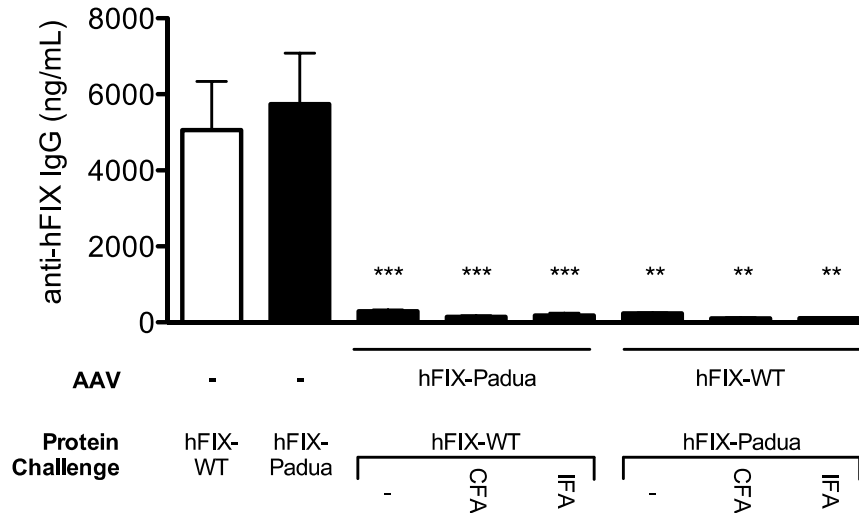


Figure 3.8 Humoral responses to hFIX-Padua following challenge. 10-15 weeks after administration of 5×10^{10} vg/kg AAV8-hFIX-R338L or AAV-hFIX-WT, HB mice were assessed for development of anti-hFIX IgG antibodies following subcutaneous challenges with 2 ug per mouse of recombinant hFIX-WT or hFIX-Padua with protein alone, protein with CFA, and protein with IFA. Untreated HB mice challenged with protein alone served as a positive control. ** $p < 0.01$; *** $p < 0.001$.

such as vector capsid, excluding the possibility of a general state of defective immune responses.

Murine Studies

Lack of immune responses to hFIX-Padua in HB mice by AAV liver gene therapy

Adult HB mice (n=9-11 mice/group) received injections of AAV encoding either hFIX-Padua or hFIX-WT resulting in similar circulating FIX plateau antigen levels (724 ± 116 ng/ml and 850 ± 96 ng/ml, respectively). Mice expressing hFIX-WT received recombinant hFIX-Padua protein and conversely, mice expressing hFIX-Padua were

challenged with hFIX-WT protein. Initial challenges started at week 10 post vector administration, followed by an additional dose every week for three weeks. No antibody to hFIX was detected by anti-hFIX total IgG (**Figure 3.8**) or Bethesda assay (data not shown). Further challenges with hFIX-Padua or hFIX-WT in CFA or in IFA failed to trigger immune responses to either hFIX forms (**Figure 3.8**). In contrast, naïve HB mice (n=4-5) develop antibodies to hFIX upon exposure to protein alone, even without adjuvant (**Figure 3.8**). Thus, upon stringent immunologic challenges with reciprocal protein no antibody formation was detected in these mice suggesting that the immunogenicity of FIX-Padua is comparable to that of FIX-WT.

Long-term expression and toxicity of FIX-Padua is comparable to FIX-WT

To assess whether expression of hFIX-Padua is associated with abnormal activation of coagulation or early death, hemostatically normal C57Bl/6 male mice received increasing vector doses encoding hFIX-Padua or hFIX-WT. As expected, hFIX specific activity was 7-10 fold-higher in the hFIX-Padua compared to hFIX-WT expressing mice (data not shown). We grouped mice expressing FIX-Padua or FIX-WT according to the FIX activity at low (100-230%) and high (480-2020%) cohorts. A time course of TAT complex levels show no difference between the FIX forms and no increase in TAT levels over time (**Figure 3.9A-B**). D-dimer levels increased as a function of time only for the low dose cohorts of hFIX-WT (p=0.0002) and hFIX-Padua (p=0.006), but there was no difference in D-dimer levels between the two FIX variants (**Figure 3.9C-D**). No changes in D-dimer levels were observed over time for the control group. Kaplan-Meier survival

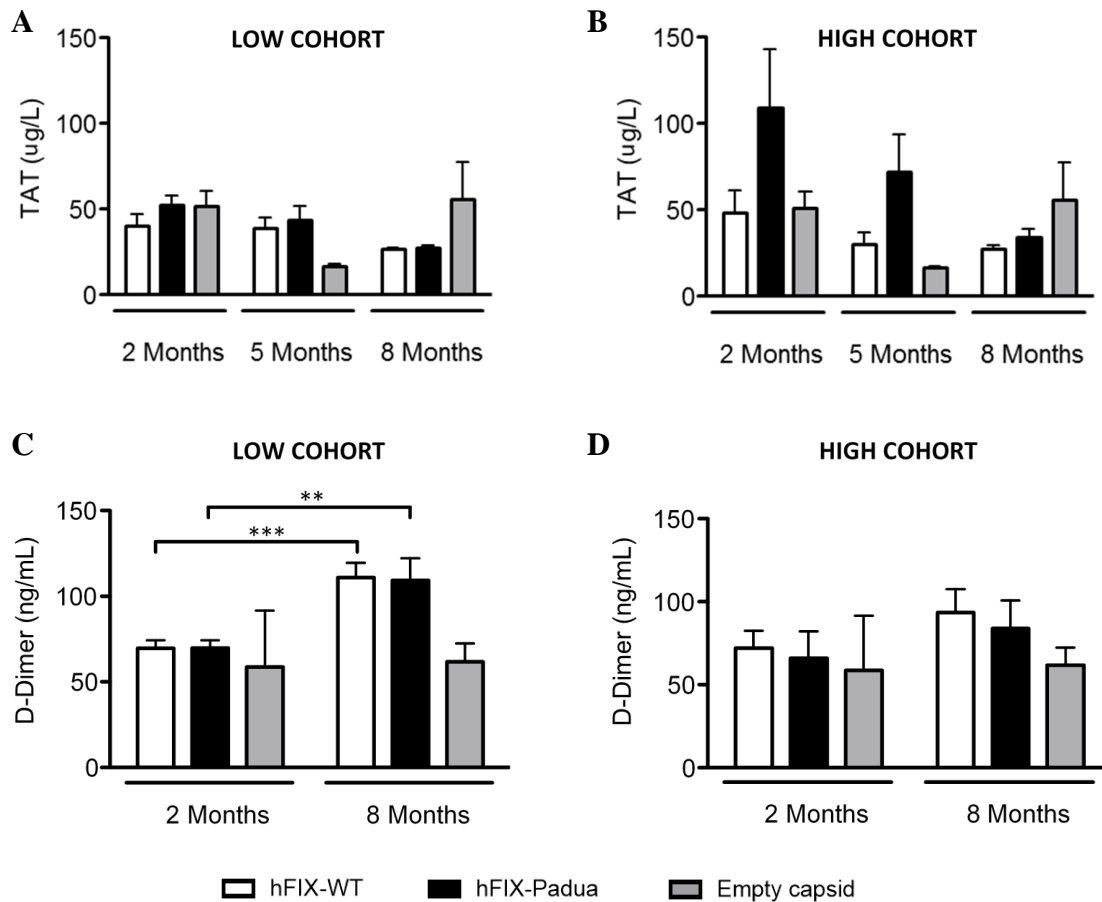


Figure 3.9 Markers of coagulation activation in wild type C57BL/6 mice expressing hFIX-WT and hFIX-Padua at supraphysiologic levels following AAV. Mice expressing hFIX-WT, white bars, and hFIX-Padua, black bars, at levels resulting in (A,C) 100-230% activity and (B,D) 480-2020% activity were assessed for (A,B) thrombin anti-thrombin complexes (TAT) and (C,D) D-dimers as markers of coagulation 2, 5 (TAT only) and 8 months following vector administration. At no point were there statistically significant differences between the hFIX-WT and hFIX-Padua expressing groups. Grey bars, empty capsid treated control animals with normal FIX levels.

analysis showed no difference between mice expressing hFIX-Padua or hFIX-WT

(**Figure 3.10**). Early death was associated with supraphysiologic levels only after 8-10 months post vector injection. Moreover, data from the control group (AAV empty capsid)

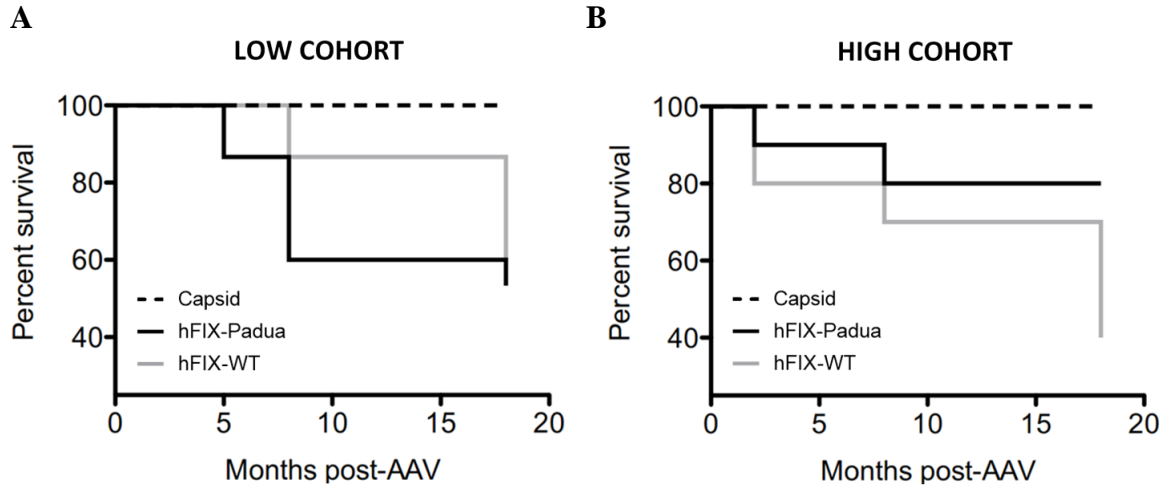


Figure 3.10 Survival of wild type C57BL/6 mice expressing hFIX-WT and hFIX-Padua at supraphysiologic levels following AAV. Mice expressing hFIX-WT, grey lines, and hFIX-Padua, black lines, at levels resulting in (A) 100-230% activity and (B) 480-2020% activity did not have different survival rates. Black dotted line, empty capsid treated control animals with normal FIX levels.

show no death during the study. Together, these findings suggest that the long-term toxicity in mice expressing a wide range of expression of hFIX-Padua is comparable of that of hFIX-WT.

IV. Discussion

One of the major safety concerns in developing novel therapies based on protein, cell, or gene therapy for hemophilia is the formation of antibodies to the missing clotting factors. The presence of these antibodies renders the replacement therapy ineffective with increased morbidity and mortality.^{209, 210, 211} Experiences with protein replacement therapy demonstrate that manufacturing procedures, mismatches between the therapeutic

protein sequence and rare haplotypes in the host, and modifications on the amino acid sequence could all influence the rates of inhibitor formation.^{180, 212, 213, 214} Here we sought to test the safety of liver-restricted expression of the cFIX-Padua variant. Severe HB dogs with an underlying *F9* gene null mutation exhibit a high risk of antibody formation upon exposure to cFIX-WT, and thus provide an excellent model to test the immune responses to the neotransgene. The data presented in HB dogs clearly demonstrate that AAV liver gene therapy ensures sustained expression of cFIX-Padua (25%-300% of normal) with high specific activity (8-12 fold) consistent with the findings in humans hemizygous for FIX-Padua.¹⁸⁹ We observed complete normalization of WBCT and TEG values concomitant with a lack of bleeding episodes over a long-term follow up in naïve HB dogs and after eradication of cFIX inhibitors in the dog Wiley.

Early-phase clinical trials for men with severe HB using AAV2 or AAV8 vectors showed that in the high dose cohort (2×10^{12} vg/kg) therapeutic levels of hFIX-WT were achieved with transient liver immune-mediated toxicity^{183, 188} controlled by a short-term course of immunosuppression¹⁸³. Here we achieved therapeutic levels of FIX activity (25-200%) well above the minimal target of 5% at vector doses of 1×10^{12} or 3×10^{12} vg/kg; therefore, it is reasonable to suggest that lower doses of AAV will be able to achieve therapeutic levels using FIX-Padua as the transgene. Surprisingly, we did not see the normally-observed AAV dose response in FIX expression. Specifically, the expression in Trex was quite poor compared to Wiley, who received the same dose, and even less than Wick, who received one third as much vector. Two things may account for this

relatively low expression. First, Trex is notably smaller than Wiley, resulting in a lower total vector dose. Secondly, the spike in Trex of GM-CSF and IL-15 (pro-inflammatory cytokines) and IL-18, IL-6, and TNF- α (Th2 and Th1 cytokines) at day 3 following vector administration is suggestive of a memory T cell response induced by AAV administration. This could indicate a prior exposure to AAV and potentially signify the presence of low titers of anti-AAV antibodies that we were unable to detect in our assay, but that could still partially inhibit AAV transduction.

In addition to high specific activities, we showed that liver expression of cFIX-Padua induced tolerance in these dogs and did not generate any anti-FIX humoral immune responses, even after challenge with cFIX-WT protein concentrate at doses associated with inhibitor formation in naïve HB dogs from this colony.¹⁹³ The expression of FIX-Padua showed no increased immunogenicity upon exposure to FIX-WT protein concentrate, which mimics the clinical scenario in which patients expressing FIX-Padua by AAV vectors (ongoing and planned clinical studies) may require protein replacement to control major bleeding or to prepare for large invasive procedures.

Notably, our data also showed that expression of AAV-cFIX-Padua was successful in eradicating inhibitory antibodies that had been triggered by previous exposure to recombinant human FIX and cross reacted to cFIX. We documented an amnestic response (peak titers ~5 BU) at early time points after AAV injection followed by eradication of the anti-cFIX antibodies and a gradual increase in the circulating levels of

fully active cFIX-Padua. The lack of immune response to the transgene was maintained after challenge with FIX-WT protein concentrate, reinforcing the strength with which AAV liver gene transfer induces transgene-specific immune tolerance. No kidney, liver or systemic toxicity was observed in this dog over a period of >2 years (ongoing observations). Of note, the inhibitory antibody (BU) to human FIX was completely eradicated, whereas residual non-neutralizing antibodies (IgG2) declined but are still detectable. This probably reflects that immune tolerance occurred to epitopes shared by canine and human FIX but not those restricted to human FIX, and thus this strategy is likely to epitope specific. The findings in this HB dog are the first demonstration of eradication of inhibitors to FIX in a large and immune competent HB model with pre-existing antibodies to FIX.

The time course for eradication of inhibitor and detection of circulating FIX levels in this dog was similar to our early data in immune tolerance induction by AAV expressing canine FVIII in hemophilia A dogs with pre-existing inhibitors (Chapter 2)²¹⁵. Despite the fact that detectable anti-FIX IgG2 resolved in 70 days, FIX activity took nearly 800 days to plateau. This suggests there were anti-FIX antibodies that our assay was unable to detect —perhaps residual poorly cross-reacting anti-hFIX, which likewise reached the limit of detection and plateau around day 800. Interestingly, Wiley's cytokine profile may reflect this. He initially showed high levels of GM-CSF, IL-15, and IL-18, markers of an inflammatory immune response, as well as IL-6, suggesting a Th2 immune response as would be expected for inhibitors. These cytokines gradually decreased over

the next 800+ days concurrent with a rise in FIX activity. In comparison, the Th1 effector cytokine TNF- α was largely undetectable in Wiley. Wick, conversely, had no detectable cytokines throughout the period he was monitored, implying Wiley's cytokine response is not in response to AAV. This, combined with the rise in FIX during this period, suggests that the cytokine profile seen in Wiley is indicative of an ongoing Th2-based, anti-FIX response that is slowly resolving. We did note elevated cytokines in Trex on day 70 following AAV administration but with no loss of cFIX expression or activity or detection of anti-cFIX antibodies. There is nothing to indicate that this isolated immune response is related to AAV administration or cFIX-Padua expression and may simply have been due to an unnoticed illness. Other than this peak and the aforementioned peak on day 3, Trex did not have elevated cytokines as was seen with the inhibitor dog Wiley.

Despite the inflammatory T cell response occurring at the early time points in Wiley, there is a striking lack of IL-2. When coupled with the expression of IL-10, this is suggestive of a possible regulatory T cell (Treg) response. In the FVIII inhibitor dog model described in Chapter 2, we likewise observed a transient increase in the pool of canine regulatory T cells (CD4⁺CD25⁺FoxP3⁺ T cells) peaking one to two weeks after vector delivery and immediately preceding the disappearance of antibodies. In Wiley, IL-10 expression rose during weeks 2 through 4, and then, interestingly, again following the protein concentrate challenge, suggesting Tregs were again required to control an anti-cFIX immune response. Also of interest is the kinetics of the IFN- γ immune

response seen in Wiley. There were two distinct peaks, one in the weeks immediately following vector administration and another following challenge with FIX protein concentrate. While normally considered to be a cytotoxic T cell and Th1 cytokine, recent papers have suggested that IFN- γ can also be transiently produced by or required for the conversion of induced Tregs (iTregs)^{216, 217}—specifically, Tregs converted from CD4⁺CD25⁻ T cells into CD4⁺CD25⁺FoxP3⁺ Tregs in the periphery. Given that we are not inducing central tolerance through the thymus, conversion of CD4⁺ effector T cells into iTregs would be the expected mechanism of Treg development in these models. The second peak in IFN- γ post-challenge, like the rise in IL-10, suggests that Tregs were again called upon to squelch an anti-cFIX immune response. This could partially explain the remaining question of the mechanism of long-lasting AAV-induced tolerance.

In this HA model, we showed that IgG2 was the predominant IgG subclass²¹⁵. The human equivalent of canine IgG2 is IgG4, and both are associated with a type 2 helper CD4⁺ T cell response¹⁹⁶. IgG4 exhibits unique properties and posttranslational modifications compared to other IgGs; notably IgG4 has poor affinity to Fc receptor and for C1q of the complement pathway, thus preventing cell and complement activation, respectively.²¹⁸ Recently, IgG4 production has been linked with activation/generation of CD4⁺ regulatory T cells and thus with a potential for down-modulated immune responses^{219, 220}. Therefore, it is possible that in the context of inhibitors to FIX in this canine model, a similar immune tolerance induction mechanism was associated with inhibitor eradication.

Data on additional dogs with pre-existing inhibitors to FIX are certainly needed to confirm this early successful observation and to more clearly define the underlying mechanism of immune tolerance. The success rates of immune tolerance induction protocols using frequent injections of FIX protein concentrates are lower than those for hemophilia A, and potential complications such nephrotic syndrome, allergic and anaphylactic reactions all hamper the overall safety^{178, 207, 221}. It is possible that AAV-based immune tolerance induction could also be indicated for a selected group of HB patients with inhibitors to FIX who are currently excluded from clinical trials.

The immune competent status of these three dogs was confirmed by the humoral responses to the vector capsid proteins. All dogs developed anti-AAV capsid IgG2 and IgG1. Similar findings were observed in humans following AAV2 or AAV8 delivery to the liver in which IgG subclasses were IgG1 and IgG2^{188, 222} or mostly IgG1, respectively¹⁸³. Thus this strategy to use AAV for the transgene-specific immune tolerance does not induce a general status of immunosuppression.

Finally, studies in mice allow us to compare the immunogenicity of FIX-Padua with FIX-WT in a stringent manner. Mice expressing FIX-Padua or FIX-WT failed to mount an immune response to the reciprocal recombinant FIX proteins, even when mixed with adjuvants to enhance immune responses.

The potential thrombogenicity of continuous expression of FIX-Padua appears to be minimal, as no evidence of pathological activation of coagulation or clinical evidence of thrombosis was noted in any of the three dogs with a cumulative observation of ~ 6 years, despite Wiley's high FIX activity levels. Together with the lack of thrombosis in another group of three HB dogs expressing FIX-Padua following AAV delivery to skeletal muscle for a cumulative 5 years²⁰⁰, there is no evidence of thrombosis in a total of 6 dogs for a combined 11 year-period, suggesting a very low risk for such a complication. This is expected considering that thrombosis occurred only in FIX-Padua patients with activity levels >700% of normal, which is significantly higher than the FIX levels in these dogs, and not in a heterozygous patient with 300-400% activity¹⁸⁹.

The comparable safety of FIX-Padua to FIX-WT is further supported by longitudinal studies in mice expressing FIX-Padua or FIX-WT, which showed similar profiles of activation of coagulation and survival rates. These data are agreement with previous short-term gene therapy studies in mice²²³. The main determinant of pathological coagulation and death rates was the supraphysiologic levels of either FIX forms that occur in a time-dependent manner, i.e. after 8-10 months post vector injection, thus providing more stringent risk assessment than short-term studies. These data suggest that the safety of continuous expression of FIX-Padua does not differ from the FIX-WT expression and, thus FIX-Padua is not inherently more thrombogenic per se, but at extremely high levels behaves in a similar fashion as FIX-WT.

Collectively the efficacy and safety data from dogs and mice expressing FIX-Padua form the basis for translational studies. The safe immunological profile of FIX-Padua in the inhibitor-prone dogs would allow the inclusion of HB patients with *F9* gene null mutations in gene therapy clinical trials.

CHAPTER 4

ACTIVATED PROTEIN C'S ANTI-METASTATIC PROPERTIES ARE INDEPENDENT OF ANTICOAGULATION AND CYTOPROTECTION

I. Introduction

Activated protein C (aPC) is a vitamin-K dependent serine protease with anticoagulant and cytoprotective functions. Thrombin generated by the coagulation cascade complexes with thrombomodulin (TM) and cleaves zymogen protein C (zyPC) bound to the endothelial protein C receptor (EPCR), converting it to aPC²²⁴. aPC, in conjunction with its cofactor protein S (PS)^{224, 225}, can then cleave FVa and FVIIIa, inactivating them and inhibiting the clotting cascade.²²⁶

aPC also has cytoprotective and anti-inflammatory effects on the endothelium through protease activated receptor 1 (PAR-1) and downstream sphingosine-1 phosphate receptor 1 (S1P₁) mediated cell signaling^{227, 228, 229}. This results in anti-apoptotic signals, downregulation of pro-inflammatory cytokines and receptors, and enhanced barrier function in the vascular endothelium²²⁷.

In addition to its anticoagulation and cytoprotective functions, aPC has been shown to have anti-metastatic effects both *in vitro* and *in vivo*^{230, 231}. Coagulation and thrombin generation are known to enhance metastasis^{232, 233}, while hirudin, a potent inhibitor of

thrombin, reduces metastasis^{234, 235}, suggesting that aPC's anticoagulant activity may be responsible for its ability to modulate cancer progression. Conversely, previous work has indicated that endogenous aPC's anti-metastatic properties stem, instead, from its cytoprotective effect mediated through the PAR-1/S1P₁ pathway²³¹. However, the mechanism of action of exogenous aPC's ability to reduce tumor metastasis even further have not been explored. Here, we examine the ability of elevated levels of aPC following gene therapy to greatly reduce the number of pulmonary metastatic lesions in the B16F10 murine melanoma model and attempt to ascertain if protection against cancer progression is due to the anticoagulant or the cytoprotective functions of aPC.

II. Materials and Methods

Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee at the Children's Hospital of Philadelphia. Eight-week-old, male C57Bl/6 mice were obtained from Jackson Laboratories. Protein C-deficient mice on the C57Bl/6 background²³⁶ were generously provided by the laboratory of Francis Castellino at the University of Notre Dame and were maintained at the Children's Hospital of Philadelphia according to institutional guidelines.

Cell culture

B16F10 murine melanoma cells (ATCC) were cultured in 1:1 DMEM/F12 (Gibco) supplemented with 10% fetal bovine serum (Gibco), 1% antibiotic-antimycotic

(penicillin-streptomycin-Fungizone, Gibco), and 1% L-glutamine at 37°C with 5% CO₂. Single cell suspensions were prepared for injection by washing cells 3 times with phosphate buffered saline (PBS) followed by 3 µM EDTA and 3 more PBS washes. Cells were then filtered, counted, resuspended in PBS, and stored on ice until injection.

Recombinant AAV vector

Production of AAV serotype 8 vectors was carried out by the Center of Cellular and Molecular Therapeutics' vector core at the Children's Hospital of Philadelphia as previously described²³⁷. The expression cassette contained wild type or mutant murine protein C with or without a PACE/Furin cleavage site motif encoded 3' of the activation peptide (proteins with the PACE/Furin site added are cleaved intracellularly and secreted as activated protein C) behind three copies of the liver-specific ApoE enhancer region and the liver-specific human alpha-1 anti-trypsin (hAAT) promoter²³⁷. Null vector consisted of human factor IX with an early stop codon behind the same promoter/enhancer.

Pulmonary metastasis model

1 x 10¹¹ vg/kg to 1 x 10¹⁴ vg/kg (5 x 10¹³ vg/kg for all experiments where dose was not specified) of AAV8 in 200 µL PBS was injected intravenously via lateral tail vein in mice on a C57Bl/6 background. Three weeks later, blood was collected via tail bleeds into 3.8% sodium citrate, and plasma was removed and frozen for later analysis. 2.5 x 10⁵ B16F10 cells resuspended in 200 µL PBS (1.25 x 10⁶ cells/mL) were injected

intravenously via lateral tail vein as previously described²³⁸. After 21 days mice were sacrificed by CO₂ asphyxiation, blood was collected from the vena cava into 3.8% sodium citrate, and lungs were perfused with 4% paraformaldehyde via the trachea prior to removal and storage in 4% paraformaldehyde at 4°C. Lung lobes were then separated and tumor foci visible with the naked eye were quantified by two independent, blinded observers.

Quantification of murine aPC and zyPC

Murine aPC plasma levels were assayed by capture ELISA as previously described^{239, 240}. Plates were coated with monoclonal anti-maPC capture antibody 1582 provided by Charles Esmon and blocked with 1% BSA/PBS. Samples were diluted 1:4 in blocking buffer and incubated overnight at 37°C; standards were diluted recombinant murine aPC, also provided by Charles Esmon. Plates were developed using the Spectrozyme PCa chromogenic substrate (American Diagnostica).

Murine zyPC levels were determined one of two ways. In the first, zyPC was activated with Protac (American Diagnostica) and assayed as above but using Spectrozyme aPC (American Diagnostica) as the chromogenic substrate. Alternatively, a traditional sandwich ELISA was performed using monoclonal antibody AMPC-9071 rat anti-murine PC (Haematologic Technologies, Inc) at 10 µg/mL in PBS to coat and 2% BSA/0.05% Tween-20/PBS to block a 96 well-plate. Standards were recombinant murine zyPC provided by Giulia Pavani and Paris Margaritis diluted in 1:4 human protein C-deficient

plasma (George King Bio-Medical, Inc):blocking buffer and samples were also diluted 1:4 in blocking buffer. Polyclonal sheep anti-murine PC PAMPC-S (Haematologic Technologies, Inc) conjugated with HRP diluted 1:600 in blocking buffer served as the secondary antibody. Plates were developed with OPD/hydrogen peroxide/sodium citrate solution.

Activated partial thromboplastin time (aPTT)

aPTTs were performed by incubating 50 μ L sample plasma with 50 μ L TriniCLOT Automated aPTT reagent for 3 minutes at 37°C, following by the addition of 50 μ L of 25 mM calcium chloride and measuring time to clot formation with a fibrometer. Plasma from hemostatically normal C57BL/6 mice typically clots in 27.3 to 32.4 seconds (30.1 ± 1.88 seconds on average) as previously reported by members of our group²⁴¹.

Tail clip assay

Tail clip assays were performed as previously described²⁴¹. Briefly, tails were placed in 37°C normal saline for two minutes and then transected at a 3 mm diameter. Tail was immediately placed in 14 mL 37°C normal saline and allowed to bleed/clot freely for 10 minutes. Tail was then removed and sutured. Blood was pelleted, then lysed in 6 mL lysis buffer, and blood loss was quantified by measuring absorbance of 575 nm light as previously described²⁴².

Statistical analysis

Statistics were run using GraphPad Prism (GraphPad Software Inc). Graphs indicate the mean plus and minus the standard deviation. Unpaired, one-tailed Welch's *t*-tests were used to compare treated groups to PBS control mice, and unpaired, two-tailed Welch's *t*-tests were used to compare variant treated groups to positive control wild type a/zyPC treated groups. *P* values <0.05 were considered statistically significant.

III. Results

Dose-dependent protection against metastasis with activated protein C

Wild type C57Bl/6 mice were administered 1×10^{12} to 1×10^{14} vg/kg AAV8 encoding murine aPC behind a liver-specific promoter enhancer. Mice receiving the lowest dose of 1×10^{12} vg/kg expressed 7.29 ± 3.0 ng/mL aPC (~4 fold over endogenous levels), which was not enough to protect against tumor metastasis in the B16F10 melanoma model (**Figure 4.1**). However, mice receiving the mid dose, 1×10^{13} vg/kg, and the high dose, 1×10^{14} vg/kg, expressed aPC at levels ~7 fold and ~28 fold over endogenous, respectively, and showed an increasing reduction in the number of pulmonary tumor foci compared to control mice administered PBS in lieu of AAV (**Figure 4.1**).

Determining metastasis with activated protein C anticoagulant-deficient variants

aPC-L38D has a missense mutation in the Gla domain at the amino acid responsible for PS binding, ablating PS cofactor function²⁴³ and slightly reducing anticoagulant activity compared to wild type aPC at high doses (**Figure 4.2A**). aPC-L38D retains the capacity

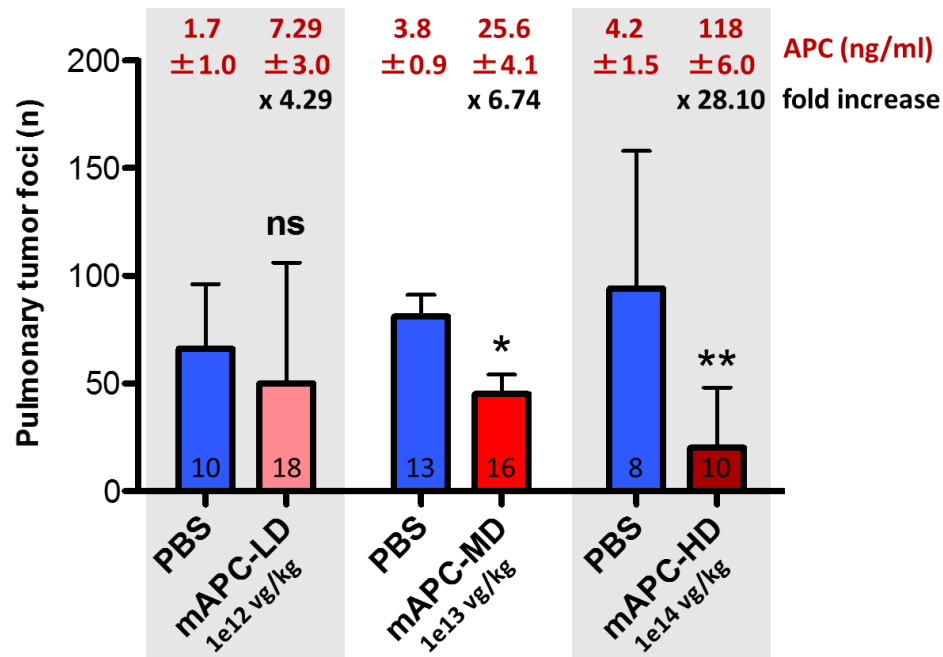


Figure 4.1 aPC expression reduces pulmonary tumor formation in a dose-dependent manner. Mice were administered 1×10^{12} to 1×10^{14} vg/kg AAV8-hAAT-maPC or PBS intravenously three weeks prior to intravenous injection of 2.5×10^5 murine B16F10 cells. Lungs were removed 21 days later and pulmonary tumor foci visible to the naked eye were quantified. Significance is comparing the treated group to the control group for each dose experiment. Red values over each bar indicate the average level \pm SD of maPC as determined by capture ELISA. Small bold black value indicate the fold increase of maPC levels in treated group compared to control group for each dose experiment. Number at the bottom of each bar indicates the number of mice in that group. * $p < 0.05$; ** $p < 0.01$; error bars indicate SD.

to bind to EPCR and cytoprotective signaling via the PAR-1 pathway²⁴³. It also retains the anti-metastatic properties of wild type aPC, significantly reducing the number of pulmonary tumors formed in the B16F10 metastasis model compared to PBS controls, with no statistical difference compared to the number of tumors in wild type aPC-expressing mice (**Figure 4.2C**). aPC-5A, on the other hand, consists of 5 amino acid

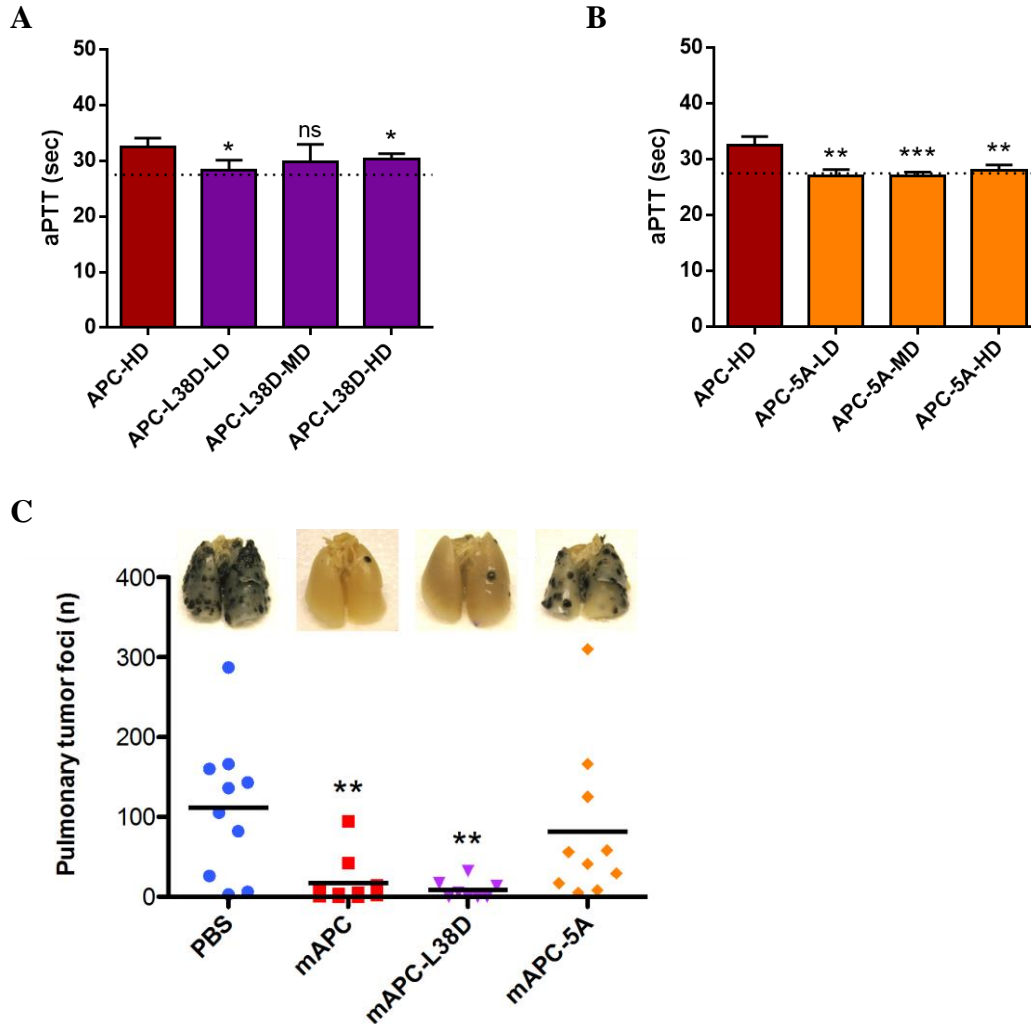


Figure 4.2 Coagulation-deficient aPC-L38D retains the anti-metastatic protective effect, but cytoprotective aPC-5A does not. (A,B) Groups of five mice were administered 5×10^{13} vg/kg AAV8-maPC (red bars) or 1×10^{12} (low dose, LD), 1×10^{13} (mid dose, MD), or 5×10^{13} (high dose, HD) vg/kg AAV8-maPC-L38D (purple bars) or AAV8-maPC-5A (orange bars). Citrated plasma was then collected and run in an aPTT. Horizontal dotted line indicates average clotting time of wild type mice. Significance indicates differences between the indicated group and the AAV8-maPC group. (B) Mice were administered 5×10^{13} vg/kg AAV8-maPC, -maPC-L38D, -maPC-5A or PBS intravenously three weeks prior to intravenous injection of 2.5×10^5 murine B16F10 cells. Lungs were removed 21 days later and pulmonary tumor foci visible to the naked eye were quantified. Significance is comparing the treated groups to the PBS control group. Horizontal bars indicate mean. Photographs are of a representative set of lungs. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant

changes in the protease domain, RR229-230AA and KKK191-193AAA^{244, 245}, which leads to near complete loss of anticoagulant function (**Figure 4.2B**) but, again, intact cytoprotective effects. This has proven to be beneficial in sepsis models, as anti-septic cytoprotective function is retained with a greatly reduced risk of bleeding. However, the 5A mutations also render aPC unable to protect against tumor metastasis, with no statistical difference in the number of tumors formed in the treated animals versus PBS controls (**Figure 4.2C**).

In order to further characterize the loss of metastasis protection in the aPC-5A variant, we divided the five mutations into two, making the aPC-3A (KKK191-193AAA) mutant and the aPC-2A (RR229-230AA) mutant, and tested their effectiveness in the B16F10 metastasis model. Murine aPC-2A, -3A, and -5A have been shown to retain just 33, 25, and 8% of normal aPC anticoagulant function, but have no loss in cytoprotective function²⁴⁶. Interestingly, the more anticoagulant function retained, the better the aPC variants were able to reduce tumor metastasis (**Figure 4.3B**).

Dose-dependent protection against metastasis with zymogen protein C

With loss of anticoagulant function having no negative impact on anti-metastatic function of aPC in one variant (L38D) and complete ablation of anti-metastatic function in another (5A), we endeavored to determine if the loss of protection seen with the aPC-5A, -3A and -2A variants was caused by the loss of anticoagulation or if it was merely correlative. To

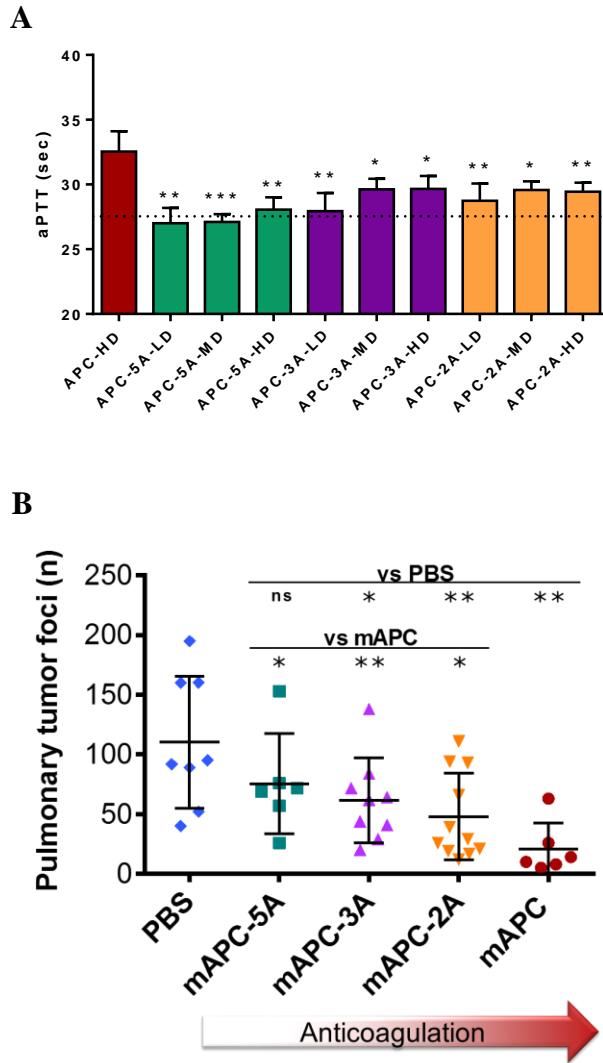


Figure 4.3 Loss of anti-metastatic effect of aPC correlates with loss of anticoagulant function in aPC-2A, -3A and -5A mutants. (A) Groups of five mice were administered 5×10^{13} vg/kg AAV8-maPC (red bars) or 1×10^{12} (low dose, LD), 1×10^{13} (mid dose, MD), or 5×10^{13} (high dose, HD) vg/kg AAV8-maPC-5A (green bars), AAV8-maPC-3A (purple bars) or AAV8-maPC-2A (orange bars). Citrated plasma was then collected and run in an aPTT. Horizontal dotted line indicates average clotting time of wild type mice. Significance indicates differences between the indicated group and the AAV8-maPC group. (B) Mice were administered 5×10^{13} vg/kg AAV8-maPC, -maPC-5A, -maPC-3A, -maPC-2A or PBS intravenously three weeks prior to intravenous injection of 2.5×10^5 murine B16F10 cells. Lungs were removed 21 days later and pulmonary tumor foci visible to the naked eye were quantified. Significance is comparing the treated groups to the PBS control group or the AAV-maPC group. Horizontal bars indicate mean \pm SD. * $p < 0.05$; ** $p < 0.01$; ns, not significant

do this, we examined zyPC's ability to protect against tumor metastasis in a dose-dependent manner. Zymogen PC does not have inherent anticoagulant activities (**Figure 4.4**), although there is literature supporting the idea that zyPC has cytoprotective functionality²⁴⁷. Even at high expression levels, zyPC did not prolong aPTT compared to wild type plasma, while just low doses of aPC did (**Figure 4.4**). Additionally, high

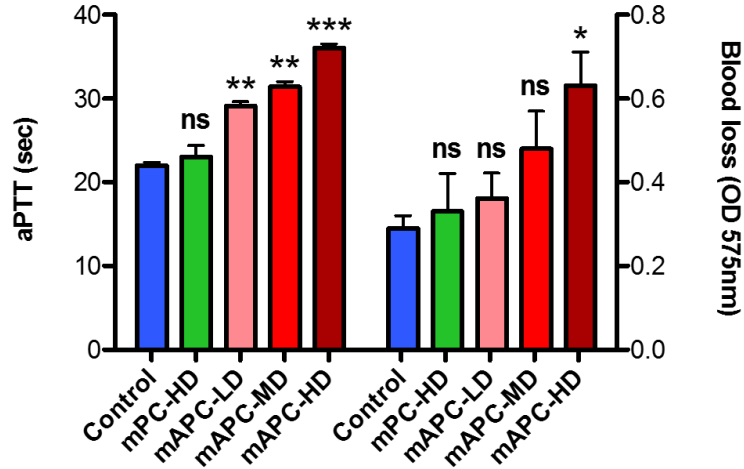


Figure 4.4 zyPC does not have anticoagulant activity. Groups of 5 mice were injected with 1×10^{14} vg/kg (HD) AAV8-mzyPC (green bars) or –maPC (dark red bars), 1×10^{13} vg/kg (MD) AAV8-maPC (red bars), 1×10^{12} vg/kg (LD) AAV8-maPC (pink bars), or PBS (blue bars). (A) Citrated plasma was collected and used in an aPTT. (B) Blood loss following a tail clip assay was determined by lysing the lost blood and reading absorbance with 575 nm light. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant.

expression levels of aPC but not zyPC increased blood loss in animals in a tail clip assay (Figure 4.4).

Wild type C57Bl/6 mice were again administered 1×10^{12} to 1×10^{14} vg/kg AAV8, this time encoding murine zyPC behind a liver-specific promoter enhancer. To measure zyPC levels, plasma was first treated with Protac to activate any zyPC, and then aPC levels were measured. Mice receiving the lowest dose of 1×10^{12} vg/kg showed levels 2-3 fold over endogenous (this fold increase was confirmed with a zyPC-specific antigen ELISA, which showed the LD group expressing zyPC at levels 2.9x over wild type), which, interestingly, was adequate to protect against tumor metastasis in the B16F10

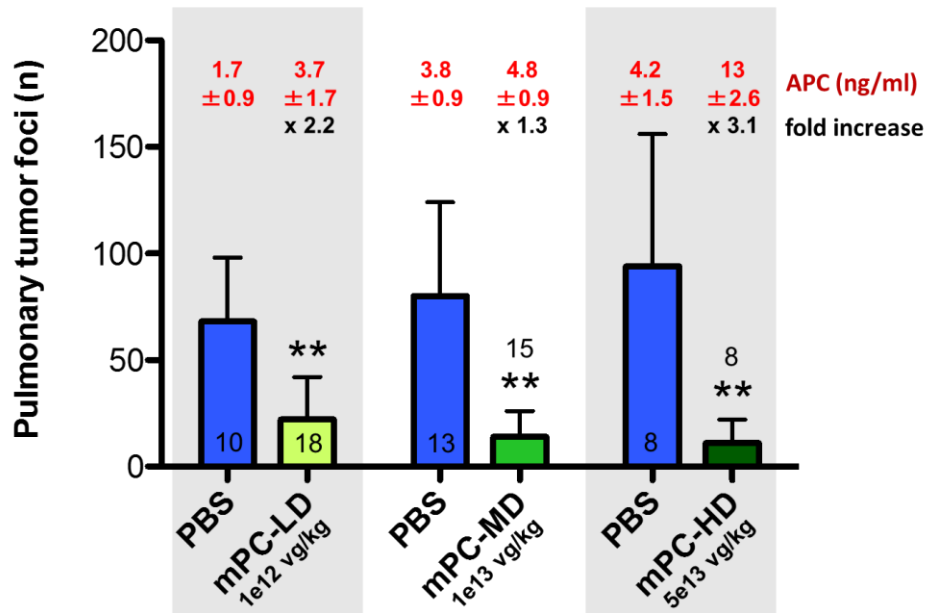


Figure 4.5 zyPC expression reduces pulmonary tumor formation at doses lower than aPC. Mice were administered 1×10^{12} to 1×10^{14} vg/kg AAV8-hAAT-mzyPC or PBS intravenously three weeks prior to intravenous injection of 2.5×10^5 murine B16F10 cells. Lungs were removed 21 days later and pulmonary tumor foci visible to the naked eye were quantified. Significance is comparing the treated group to the control group for each dose experiment. Red values over each bar indicate the average level \pm SD of maPC as determined by capture ELISA after zyPC activation with Protac. Small bold black value indicate the fold increase of maPC levels in treated group compared to control group for each dose experiment. Number at the bottom of each bar indicates the number of mice in that group. ** $p < 0.01$; error bars indicate SD.

melanoma model (**Figure 4.5**). Such protection had not been seen in the low dose of aPC vector administration, which had required the mid dose and 6-7 fold over endogenous levels before there was a statistical anti-metastatic effect (**Figure 4.1**). There was an increasing reduction in the number of pulmonary tumor foci as zyPC vector doses increased correlating with increased expression (**Figure 4.5**). An even lower dose of 1×10^{11} vg/kg AAV-mzyPC resulting in expression levels equal to just 150% endogenous

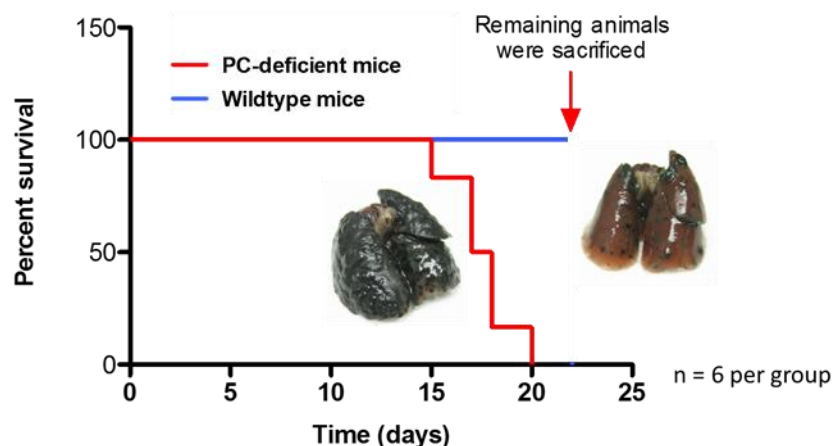


Figure 4.6 Endogenous zyPC modulates metastatic tumor formation. Untreated protein C-deficient mice expressing <15% normal zyPC levels (red line) and wild type littermate controls (blue line) were injected intravenously with 2.5×10^5 murine B16F10 cells and monitored daily. Remaining mice were sacrificed 21 days later. Lungs were removed from all mice following death. Photographs are of a representative set of lungs.

levels was inadequate to protect against metastasis (data not shown).

To further support the involvement of zyPC in preventing metastasis, we used a murine model deficient in PC. Antigen ELISAs performed by our lab show these mice express approximately 5-15% of normal zyPC levels. When injected with B16F10 cells without prior treatment, PC-deficient mice all died by day 20 while their wild type littermate controls survived for the duration of the 21 day experiment (**Figure 4.6**). Additionally, while the wild type mice had typical pulmonary tumor counts ranging from 50 to 200, the number of tumors in the lungs of the PC-deficient mice were so numerous as to be unquantifiable (see photo in **Figure 4.6**).

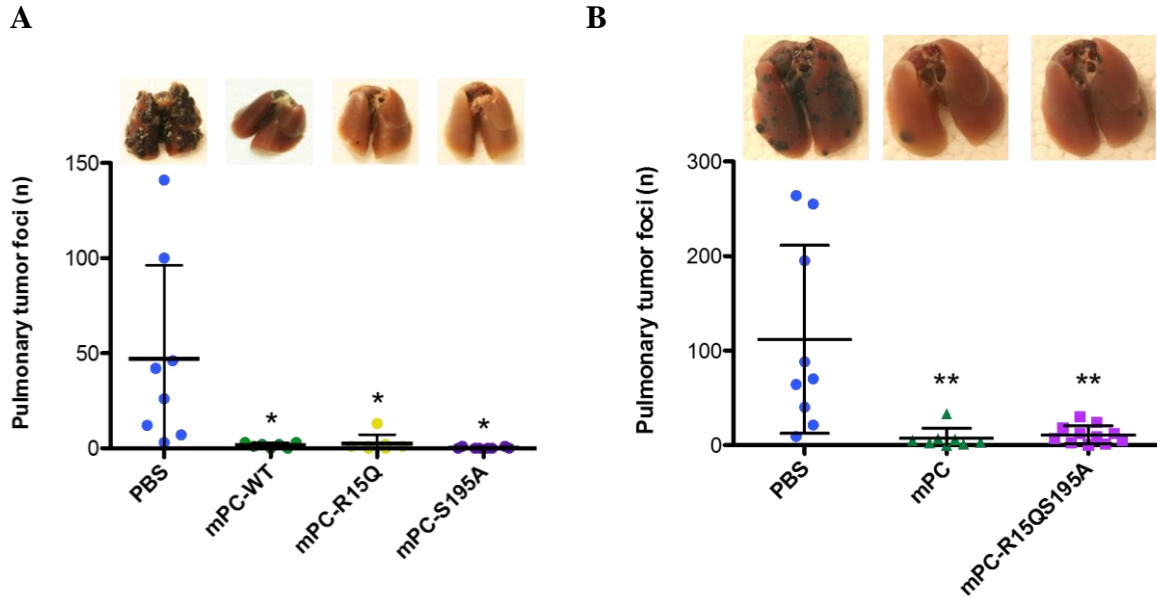


Figure 4.7 Activation- and catalytic site-deficient zyPC-R15Q and zyPC-S195A retain the anti-metastatic protective effect. Mice were administered 5×10^{13} vg/kg AAV8-mzyPC (A&B, green), -mzyPC-R15Q (A, yellow), -mzyPC-S195A (A, purple), -mzyPC-R15QS195A (B, purple) or PBS (A&B, blue) intravenously three weeks prior to intravenous injection of 2.5×10^5 murine B16F10 cells. Lungs were removed 21 days later and pulmonary tumor foci visible to the naked eye were quantified. Significance is comparing the treated groups to the PBS control group in each experiment. Horizontal bars indicate mean \pm SD. * $p < 0.05$; ** $p < 0.01$.

zyPC protection against metastasis is not a result of local conversion to aPC

While zyPC does not have any inherent anticoagulant activity, it is possible that the high levels of circulating zymogen meant an increase in aPC conversion and thus anticoagulation at sites of tumor metastasis. To explore this possibility, we used mice expressing the zyPC-R15Q mutant, which lacks the ability to dock to the thrombin-thrombomodulin complex and therefore cannot be cleaved²⁴⁸, or zyPC-S195A, a mutation of the catalytic serine in the active site. Both retained the ability to protect

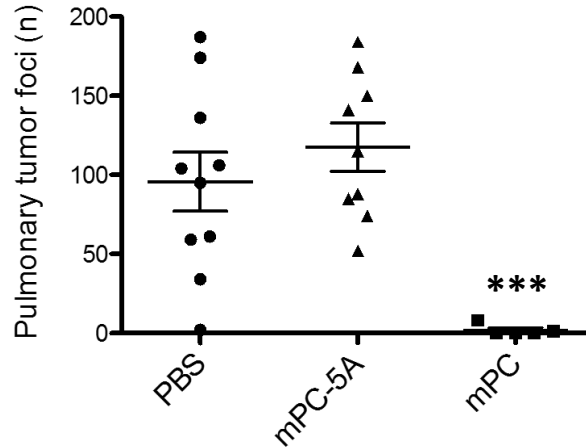


Figure 4.8 Loss of protection with 5A mutations is not due to loss of anticoagulation. Mice were administered 5×10^{13} vg/kg AAV8-mzyPC (squares), -mzyPC-5A (triangles), or PBS (circles) intravenously three weeks prior to intravenous injection of 2.5×10^5 murine B16F10 cells. Lungs were removed 21 days later and pulmonary tumor foci visible to the naked eye were quantified. Significance is comparing the treated groups to the PBS control group. Horizontal bars indicate mean \pm SD. *** $p < 0.001$.

against tumor metastasis, as did the double mutant zyPC-R15QS195A, with equal levels of protection seen with wild type zyPC (**Figure 4.7**).

Loss of protection is not due to loss of coagulation in the 5A mutant

While zyPC does not appear to require anticoagulant function in order to protect against metastasis, the loss of protection in the aPC-2A, -3A, and -5A mutants could still be due to loss of anticoagulant function if zyPC and aPC prevented metastasis through different mechanisms. To examine this, we made the 5A mutations in the zyPC, as these protease domain mutations would have no adverse effect on the already non-existent anticoagulant function of zyPC. Notably, zyPC-5A is unable to protect against tumor metastasis

(**Figure 4.8**), suggesting that the loss of anticoagulant function in the aPC-5A mutant is correlative, not causal, with the loss of metastasis protection.

Reduction of tumor metastasis with overexpression of a/zyPC is not dependent on PAR-1-mediated cytoprotection

The loss of anti-metastatic properties in the cytoprotective intact aPC-5A, -3A, -2A, and zyPC-5A mutants suggests that tumor protection with overexpression of a/zyPC is not through the cytoprotective pathway. However, we sought to further confirm this using an additional mutant and PAR-1 deficient mice.

aPC-E149A has a mutation in the protease domain, resulting in a signaling defective variant with severely reduced anti-inflammatory, anti-apoptotic, and cytoprotective functions²⁴⁹. This is despite normal binding of EPCR and cleavage of PAR-1. When mice expressing aPC-E149A were challenged with B16F10 cells, they were equally protected against pulmonary lesions as mice expressing wild type aPC (**Figure 4.9**).

To definitively determine if PC-mediated metastasis protection is PAR-1 dependent, we utilized PAR-1 knockout mice. Knockout mice expressing zyPC were protected against tumor metastasis compared to PBS control knockout mice at a similar level of protection seen in zyPC-expressing heterozygous littermate controls, which are phenotypically the same as homozygous wild type mice (**Figure 4.10A**). This is especially interesting given that PAR-1 knockouts develop less tumors than wild type mice (**Figure 4.10B**).

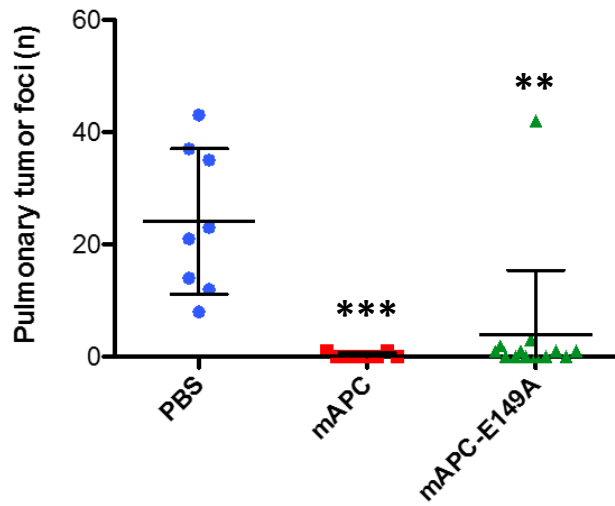


Figure 4.9 Cytoprotective-deficient aPC-E149A retains the anti-metastatic effect. Mice were administered 5×10^{13} vg/kg AAV8-maPC (red), -maPC-E149A (green), or PBS (blue) intravenously three weeks prior to intravenous injection of 2.5×10^5 murine B16F10 cells. Lungs were removed 21 days later and pulmonary tumor foci visible to the naked eye were quantified. Significance is comparing the treated groups to the PBS control group. Horizontal bars indicate mean \pm SD. ** $p < 0.001$, *** $p < 0.001$.

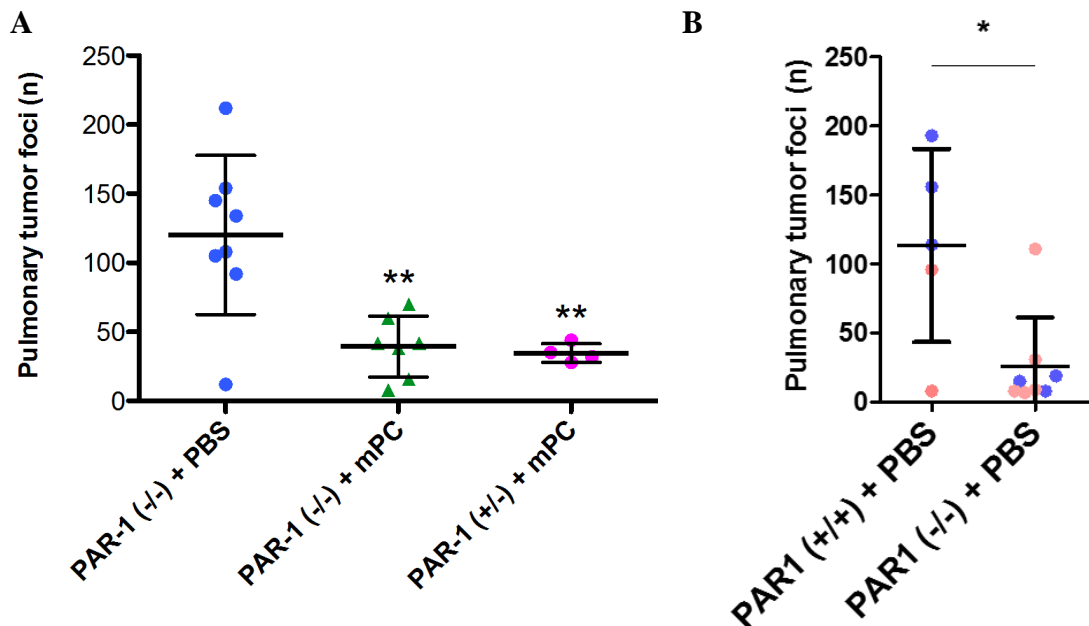


Figure 4.10 The anti-metastatic effect of zyPC is not mediated through PAR-1 cytoprotection. (A) PAR-1 knockout mice were administered 5×10^{13} vg/kg AAV8-mzyPC (green) or PBS (blue) and compared to PAR-1 heterozygous littermate controls that received 5×10^{13} vg/kg AAV8-mzyPC (purple) intravenously three weeks prior to intravenous injection of 2.5×10^5 murine B16F10 cells. (B) Untreated PAR-1 knockouts or wild type littermate controls (females, pink; males, blue) received 2.5×10^5 murine B16F10 cells i.v. In both experiments, lungs were removed 21 days later and pulmonary tumor foci visible to the naked eye were quantified. Significance is comparing the treated groups to the PBS control group in A. Horizontal bars indicate mean \pm SD. * $p < 0.05$; ** $p < 0.001$.

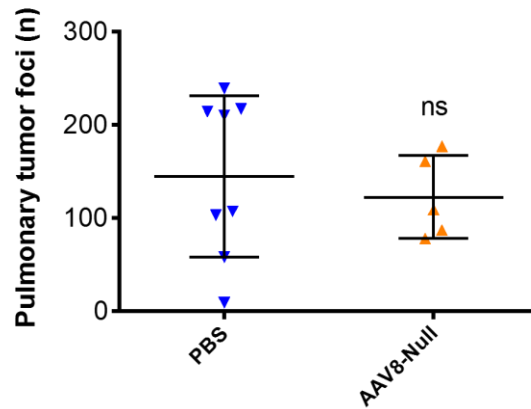


Figure 4.11 Anti-metastatic protection is not mediated through AAV alone. Mice were administered 5×10^{13} vg/kg AAV8-FIX with an early stop codon (AAV8-Null, orange) or PBS (blue) intravenously three weeks prior to intravenous injection of 2.5×10^5 murine B16F10 cells. Lungs were removed 21 days later and pulmonary tumor foci visible to the naked eye were quantified. Significance is comparing the treated groups to the PBS control group. Horizontal bars indicate mean \pm SD. ns, not significant.

AAV does not protect against tumor metastasis

To ensure that the anti-metastatic effect seen in these experiments was not due to AAV alone, mice were treated with an AAV null vector, which has an intact transgene with an early stop codon that prevents translation of protein. No protection was seen with AAV8-Null (**Figure 4.11**), which is to be expected given that the vectors expressing the 5A mutants were likewise not protective (**Figures 4.2C, 4.3B, 4.8**).

IV. Discussion

aPC is known to be protective against tumor metastasis. While experimental models targeting thrombin are known to prevent metastasis, previous work by Van Sluis, *et al.*²³¹

indicated that endogenous aPC's mechanism of protection did not involve the anticoagulation properties of aPC. Furthermore, they argued that protection was mediated through the PAR-1 cytoprotective pathway. Here, we sought to determine if the ability of aPC to reduce tumors in the context of overexpression of aPC worked through a similar mechanism.

As with Van Sluis, *et al.*, we did not see a loss of protection against metastasis with the anticoagulant defective aPC-L38D mutant. However, there was a loss of protection with the aPC-5A mutant, which has a much greater reduction in anticoagulation, and loss of protection seemed to correlate with loss of anticoagulation, as seen with the intermediary -3A and -2A mutants. However, we show here for the first time, that the zymogen PC, including an enzymatic site mutant that cannot be activated (zyPC-R15QS195A), which has no anticoagulant function, is equally protective, if not more so, and that zyPC-5A is also unable to protect against tumor metastasis. This suggests that aPC and zyPC are working through the same mechanism—one that is independent of anticoagulation—and that the loss of anticoagulation in the 5A mutants is correlative, not causal. It also suggests that the 5A mutants have lost an additional function, perhaps involving a protein-protein interaction that renders it unable to prevent metastasis. Bezhuly, *et al.*²⁵⁰ demonstrated that treating an endothelial cell monolayer with recombinant human aPC *in vitro* prevented B16F10 adhesion to and transmigration through that monolayer. This further supports the idea that zy/aPC is blocking some protein (perhaps receptor)-mediated binding and extravasation by tumor cells.

Unlike Van Sluis, *et al.*, the ability of supraphysiologic levels of zy/aPC to protect against metastasis was not dependent upon PAR-1 mediated cytoprotection. Despite having intact cytoprotective function aPC-5A, -3A, and -2A were unable to prevent tumor metastasis like wild type aPC. Additionally, the signaling deficient aPC-E149A was just as protective as the wild type aPC, and even completely knocking out PAR-1 did not prevent zyPC from protecting against metastasis. There are two main reasons this discrepancy between our work and that of Van Sluis, *et al.* could exist. First, it is likely that endogenous levels of cytoprotection are necessary for modulating metastasis. Without an effective endothelial barrier, tumor cell extravasation from the blood stream into new tissue sites might be unhindered (although, interestingly, knocking out PAR-1 has the opposite effect). However, it is possible that with the barrier intact, additional zy/aPC has no effect, as most EPCR is already occupied by zyPC and most PAR-1 signaling already cytoprotective, so that pathway might be saturated. The second explanation for the divergent results is that, like loss of anticoagulation in the 5A mutants, loss of coagulation with the antibody that renders aPC unable to protect against metastasis is correlative, not causal. Interestingly, the antibody used by those authors that targets aPC alone does not inhibit the ability of the PC pathway to protect against metastasis, it is only the antibody that prevents both zyPC and aPC from binding to the endothelium that results in loss of anti-metastatic function. While a lack of binding to EPCR and thus the endothelium would prevent cytoprotection, it is possible that this antibody is blocking zy/aPC competitive binding to an additional protein that tumor cells require access to in order to extravagate. While plausible, this latter theory cannot

explain why stimulation of the S1P₁ receptor, which is downstream of the PAR-1 signaling pathway, reverses the effect of the antibody. Thus, it is possible that the discrepancy between our data and theirs involves a combination of both scenarios.

It is notable that zyPC is as effective—if not more so—as aPC in preventing metastasis. aPC half-life is only 15 minutes, and, as the no longer on the market drug Xigris demonstrated, aPC's associated bleeding risks are potentially severe. While it is true that many cancer patients may actually benefit from aPC's anticoagulant properties, avoiding unwanted bleeds is a priority. The risk of such bleeds with zyPC-R15QS195A is essentially zero, as the zymogen has no anticoagulant function, the active site mutation is redundant, and the thrombin-TM docking site mutation prevents the mutant from ever being activated. Such a protein could be used for patients with high risk metastatic cancers, especially at the time of primary tumor resection, and would not require as frequent dosing given that zyPC has a much longer half-life (approximately 6 hours). Interestingly, doses of zyPC similar to those used here to confer protection against metastasis (~400%) have been given to healthy volunteers for a sepsis trial with no adverse effects²⁵¹. However, if the mechanism of action can be found, it is possible that a small molecule inhibitor or antibody could be developed that would have an even longer half-life with minimal side effects.

CHAPTER 5

SEARCHING FOR THE MECHANISM THROUGH WHICH PROTEIN C PREVENTS CANCER PROGRESSION

I. Introduction

In Chapter 4 we demonstrated that, despite suggestive literature, overexpression of aPC prevents tumor metastasis independent of aPC's anticoagulant and cytoprotective effects. Importantly, we also demonstrated that zyPC is as protective as aPC and more feasible as a potential drug candidate, with a longer half-life and an increased safety profile associated with reduced risk of bleeding. However, if the mechanism of action can be found, a small molecule inhibitor or antibody could be developed with even more appealing characteristics related to dosing frequency and side effects.

Our work and the *in vitro* work of Bezhuly, *et al.*²⁵² suggest that this mechanism might involve competitively binding an endothelial cell surface receptor or protein, preventing tumor cell adherence and extravasation. However, what this receptor might be is unknown, though previous work has demonstrated involvement of a few receptors in B16F10 tumor metastasis, including EPCR²⁵², PAR-4, and apoER2/LRP8²⁵³. Moreover, there are number of putative receptors that zy/aPC are thought to bind, including integrins on white blood cells and various additional platelet receptors²⁵⁴.

It is also unknown if the competitive receptor binding is the only mechanism of action. For example, it is known that thrombin has a positive effect on tumor cell growth and angiogenesis²⁵⁵, and so it is possible that zyPC can likewise inhibit these or other steps in cancer progression.

Here, we begin to investigate some of these receptors' involvement in the anti-metastatic ability of zyPC and attempt to determine which metastatic steps zyPC blocks.

II. Materials and Methods

Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee at the Children's Hospital of Philadelphia. Eight-week-old, male C57Bl/6 mice were obtained from Jackson Laboratories. NOD *scid* gamma (NSG) mice were obtained from the Children's Hospital of Philadelphia breeding core. EPCR^{lo} mice on the C57Bl/6 background were generously provided by Charles Esmon, and were maintained at the Children's Hospital of Philadelphia according to institutional guidelines.

Cell culture

B16F10 murine melanoma cells (ATCC) and 4T1 murine breast cancer cells (ATCC) were cultured in 1:1 DMEM/F12 (Gibco) supplemented with 10% fetal bovine serum (Gibco), 1% antibiotic-antimycotic (penicillin-streptomycin-Fungizone, Gibco), and 1% L-glutamine at 37°C with 5% CO₂. Single cell suspensions were prepared for injection

by washing cells 3 times with phosphate buffered saline (PBS) followed by 3 μ M EDTA and 3 more PBS washes. Cells were then filtered, counted, resuspended in PBS, and stored on ice until injection.

Tumor cell proliferation/growth assay

293 producer lines expressing either canine FIX, murine zymogen protein C, or nothing were seeded in the upper chamber of a 3 μ m pore transwell 6-well plate with the aforementioned growth media supplemented with vitamin K. Two days later, B16F10 cells were seeded into the lower half of the chamber. 24, 48, and 72 hours later, three wells of B16F10s per experimental group were counted in duplicate following treatment with Trypsin-EDTA to detach the cells.

Tumor cell adhesion assay

All wells were done in duplicate. Wells of 6-well plates were pre-coated with 10 μ g fibronectin, 6 μ g Matrigel, 10 μ g collagen or nothing in 1 mL PBS overnight at 4°C. Plates were then blocked with 1% BSA/PBS (with the exception of the control uncoated wells). 1×10^5 B16F10s that had been previously co-cultured with 293s expressing cFIX, mzyPC, or nothing were then seeded into the wells in 1 mL media (enriched with 4 μ g recombinant mzyPC for the appropriate wells). 45 minutes later, plates were washed 3 times with PBS to wash off non-adherent cells, and remaining cells were fixed for 15 minutes in 70% methanol and then stained for 15 minutes with 5% crystal violet/20% methanol. Plates were washed vigorously and repeatedly in deionized water to remove

excess crystal violet. The stain was released from the cells by adding 30% acetic acid, and color was quantified by reading absorbance at 590 nm. ODs were normalized against the uncoated, unblocked control wells of the same time (co-cultured with 293, 293-cFIX or 293-mPC) to account for small differences in the numbers of cells seeded per well.

Recombinant AAV vector

Production of AAV serotype 8 vectors was carried out by the Center of Cellular and Molecular Therapeutics' vector core at the Children's Hospital of Philadelphia as previously described²⁵⁶. The expression cassette contained wild type or mutant murine protein C with or without a PACE/Furin cleavage site motif encoded 3' of the activation peptide (proteins with the PACE/Furin site added are cleaved intracellularly and secreted as activated protein C) behind three copies of the liver-specific ApoE enhancer region and the liver-specific human alpha-1 anti-trypsin (hAAT) promoter²⁵⁶. Null vector consisted of human factor IX with an early stop codon behind the same promoter/enhancer.

Melanoma primary tumor model

5×10^{13} vg/kg of AAV8 in 200 μ L PBS was injected intravenously via lateral tail vein in mice on a C57Bl/6 background. Three weeks later, blood was collected via tail bleeds into 3.8% sodium citrate, and plasma was removed and frozen for later analysis. 1×10^4 B16F10 cells resuspended in 100 μ L PBS were injected subcutaneously on the right

flank. Tumor development was monitored, and after primary tumors were palpable in most mice (day 19) they were measured with calipers. They were measured again on day 25. After 26 days mice were sacrificed by CO₂ asphyxiation, blood was collected from the vena cava into 3.8% sodium citrate, and primary tumors were removed and weighed.

Melanoma pulmonary metastasis model

5 x 10¹³ vg/kg of AAV8 in 200 µL PBS was injected intravenously via lateral tail vein in mice on a C57Bl/6 background (or NSG mice). Three weeks later, blood was collected via tail bleeds into 3.8% sodium citrate, and plasma was removed and frozen for later analysis. 2.5 x 10⁵ B16F10 cells—unless otherwise indicated—resuspended in 200 µL PBS (1.25 x 10⁶ cells/mL) were injected intravenously via lateral tail vein as previously described²⁵⁷. To block zyPC binding to EPCR, mice were first injected with 100 µg monoclonal rat anti-mouse EPCR blocking antibody (RMEPCR1560)²⁵⁸ or an isotype control (both provided by Charles Esmon) in 0.5 mL PBS intraperitoneally 1 hour prior to B16F10 injection. After 21 days mice were sacrificed by CO₂ asphyxiation, blood was collected from the vena cava into 3.8% sodium citrate, and lungs were perfused with 4% paraformaldehyde via the trachea prior to removal and storage in 4% paraformaldehyde at 4°C. Lung lobes were then separated and tumor foci visible with the naked eye were quantified by two independent, blinded observers.

Breast cancer pulmonary metastasis model

5 x 10¹³ vg/kg of AAV8 in 200 µL PBS was injected intravenously via lateral tail vein in mice on a Balb/c background. Three weeks later, blood was collected via tail bleeds into 3.8% sodium citrate, and plasma was removed and frozen for later analysis. 1 x 10⁴ 4T1 cells in 100 µL PBS were injected subcutaneously on the right flank. As mice reached primary tumor endpoints (4-5 weeks later), they were sacrificed by CO₂ asphyxiation, blood was collected from the vena cava into 3.8% sodium citrate, and lungs were perfused with India ink solution (15% v/v India Ink in dH₂O) via the trachea prior to removal and storage in Fekete's solution at 4°C. Lung lobes were then separated and white tumor foci visible with the naked eye were quantified by two independent, blinded observers.

Quantification of murine zyPC

Murine zyPC levels were determined with a traditional sandwich ELISA performed using monoclonal antibody AMPC-9071 rat anti-murine PC (Haematologic Technologies, Inc) at 10 µg/mL in PBS to coat and 2% BSA/0.05% Tween-20/PBS to block a 96 well-plate. Standards were recombinant murine zyPC provided by Giulia Pavani and Paris Margaritis diluted in 1:4 human protein C-deficient plasma (George King Bio-Medical, Inc):blocking buffer and samples were also diluted 1:4 in blocking buffer. Polyclonal sheep anti-murine PC PAMPC-S (Haematologic Technologies, Inc) conjugated with HRP diluted 1:600 in blocking buffer served as the secondary antibody. Plates were developed with OPD/hydrogen peroxide/sodium citrate solution.

Flow cytometry

Human embryonic kidney 293 cells (negative control), murine Mile Sven 1 endothelial (MS-1) cells (positive control provided by Stephen Santoro), B16F10 cells, and 4T1 cells were harvested from monolayers with 3 μ M EDTA, stained with rat anti-murine EPCR conjugated with FITC (StemCell Technologies), and histograms were generated with a BD Canto flow cytometer and FlowJo software (Treestar, Ashland, OR).

Statistical analysis

Statistics were run using GraphPad Prism (GraphPad Software Inc). Graphs indicate the mean plus and minus the standard deviation. Unpaired, one-tailed Welch's t-tests were used to compare treated groups to PBS control mice, and unpaired, two-tailed Welch's t-tests were used to compare variant treated groups to positive control wild type zyPC treated groups. P values <0.05 were considered statistically significant.

III. Results

Effect of zyPC on B16F10 tumor cell growth and adhesion

B16F10 cells grown in the presence of a mzyPC-producing 293 line had neither their growth (**Figure 5.1A**) nor their adhesion to collagen, fibrinogen, Matrigel, or BSA-blocked tissue culture wells (**Figure 5.1B**) negatively affected compared to B16F10 cells grown only in the presence of a non-producing 293 line. A 293 line producing cFIX was used as an additional negative control, though interestingly adherence of the B16F10s was greatly improved in the presence of cFIX.

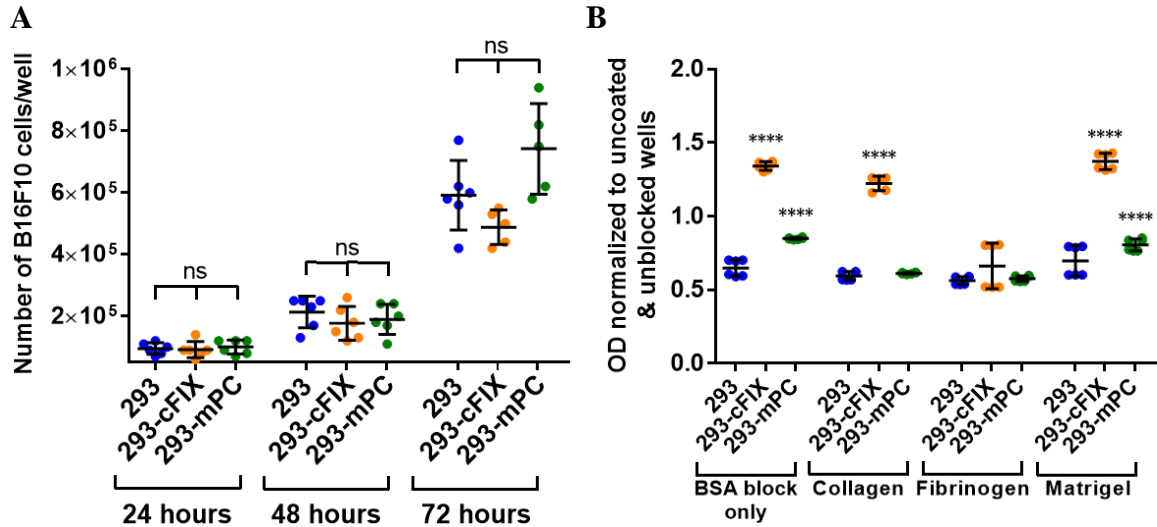


Figure 5.1 zyPC does not negatively affect B16F10 tumor cell growth or adhesion *in vitro*. (A) B16F10 melanoma cancer cells were seeded in the bottom of a 6-well transwell plate with 3 μ m pores, with 293 producer cells stably expressing canine FIX (293-cFIX), murine zyPC (293-mPC), or nothing (293), and grown for 24, 48, or 72 hours before counting. Wells were plated in triplicate, and each well was counted in duplicate. (B) Plates were pre-coated with collagen, fibrinogen, Matrigel or nothing, then blocked with 1% BSA (except for uncoated normalization wells) before plating B16F10s that had been grown in a transwell system with 293 producer cells stably expressing canine FIX (293-cFIX), murine zyPC (293-mPC), or nothing (293) for more than one week. Recombinant mzyPC was also spiked into the wells with B16F10s that had been grown with 293-mPC cells at a concentration of 4 μ g/well. 45 minutes later, wells were washed and adherent cells were stained with crystal violet. Retained stain was released with acetic acid and measured with a spectrophotometer at 590 nm. Wells were done in duplicate and read in triplicate and then normalized against uncoated, unblocked wells. ns, not significant.

B16F10 growth was likewise not inhibited *in vivo*. Mice expressing mzyPC and PBS control mice had a primary B16F10 tumor seeded subcutaneously in their right flank. Primary tumor growth was monitored with calipers, and tumors were weighed at the end of the experiment. There was no difference in the growth of the primary tumor in animals overexpressing mzyPC and wild type animals.

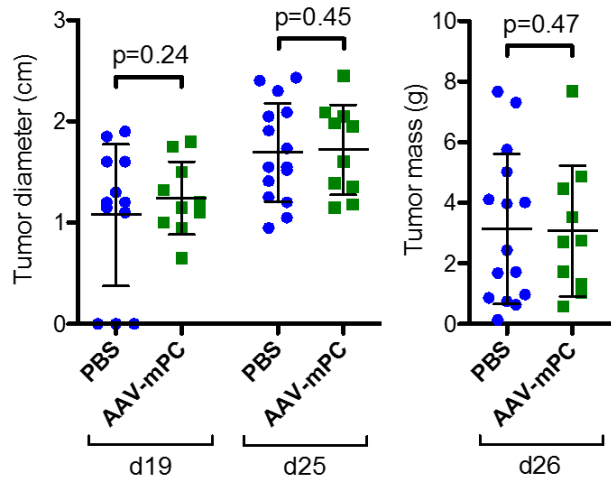


Figure 5.2 zyPC does not affect primary tumor growth *in vivo*. Mice were administered 5×10^{13} vg/kg AAV8-mzyPC (green) or PBS (blue) intravenously three weeks prior to subcutaneous injection of 1×10^4 murine B16F10 cells. Primary tumors were measured with calipers on days 19 and 25, and then removed and weighed on day 26. Significance is comparing the treated groups to the PBS control group. Horizontal bars indicate mean \pm SD.

EPCR is not required for zyPC protection against metastasis

Previous work has shown that overexpression of EPCR decreases tumor metastasis in the B16F10 model, suggesting that PC-mediated protection is EPCR-dependent, although it was never directly shown. In an attempt to address this question, we first utilized an anti-EPCR antibody (RMEPCR1560) that prevents zyPC binding to EPCR. Mice expressing zyPC and wild type mice were injected with blocking antibody, an isotype control, or PBS intraperitoneally 1 hour prior to B16F10 intravenous injection. Wild type mice injected with the antibody did not develop tumors differently than wild type mice injected intraperitoneally with PBS, suggesting that the anti-EPCR antibody did not have an effect on metastatic pulmonary tumor formation. zyPC-expressing mice injected with the anti-

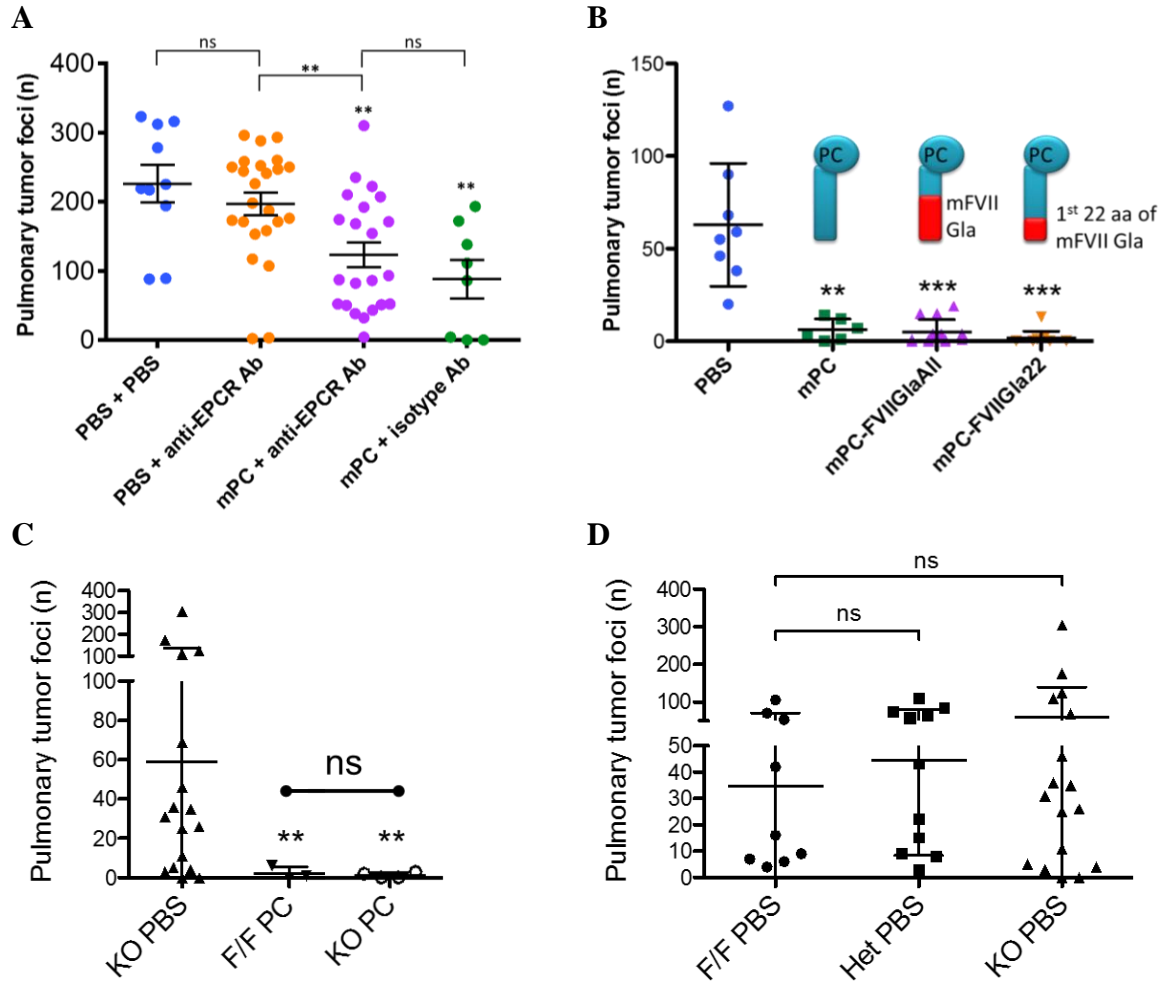


Figure 5.3 zyPC anti-metastatic properties are not dependent on interaction with EPCR. (A) Mice were administered 5×10^{13} vg/kg AAV8-mzyPC (purple, green) or PBS (blue, orange) intravenously. Three weeks later mice were injected intraperitoneally with PBS (blue), an anti-EPCR blocking antibody (orange, purple), or an isotype control antibody (green) one hour prior to intravenous injection of 2.5×10^5 murine B16F10 cells. Lungs were removed 21 days later and pulmonary tumor foci visible to the naked eye were quantified. Significance is comparing the treated groups to the PBS + PBS control group, except where indicated with bars. (B) Mice were administered 5×10^{13} vg/kg AAV8-mzyPC (green), -mzyPC with the entire mFVIIIa Gla domain (mPC-FVIIIGlaAll, purple), -mzyPC with the first 22 amino acids of the mFVIIIa Gla domain (mPC-FVIIIGla22, orange), or PBS (blue) intravenously three weeks prior to intravenous injection of 2.5×10^5 murine B16F10 cells. Lungs were removed 21 days later and pulmonary tumor foci visible to the naked eye were quantified. (C) EPCR conditional knockout mice (EPCR^{lo}) were administered 5×10^{13} vg/kg AAV8-mzyPC (open circles) or PBS (upward triangles) and compared to EPCR-floxed homozygous littermate controls that received 5×10^{13} vg/kg AAV8-mzyPC (downward triangles) intravenously three

weeks prior to intravenous injection of 2.5×10^5 murine B16F10 cells. (D) Untreated EPCR^{lo}, heterozygous, or floxed mice received 2.5×10^5 murine B16F10 cells i.v. Lungs were removed 21 days later and pulmonary tumor foci visible to the naked eye were quantified. Significance is comparing the treated groups to the PBS control group unless otherwise indicated. Horizontal bars indicate mean \pm SD. ** $p < 0.001$; *** $p < 0.001$; ns, not significant.

EPCR antibody had significantly less tumors compared to wild type mice injected intraperitoneally with PBS or blocking antibody. The reduction in tumor formation was similar to that seen in zyPC-expressing mice injected intraperitoneally with an isotype control antibody (**Figure 5.3A**).

In order to confirm the results seen with the blocking antibody, we made zyPC mutants that would not bind to EPCR. zyPC-EPCR binding is mediated through the Gla domain—specifically the first 8 amino acids, with the first 22 being associated with phospholipid membrane binding. Therefore, mice expressing a wild type zyPC or a zyPC fusion molecule where either the entire Gla domain or just the first 22 amino acids of the Gla domain was swapped with mFVIIIa, which is known to not bind to mEPCR^{259, 260}, were used in the B16F10 model. Both fusion molecules were equally protected against metastatic pulmonary tumor formation at comparable levels to wild type zyPC (**Figure 5.3B**).

Finally, we utilized EPCR^{lo} mice to truly eliminate the involvement of EPCR. These mice are generated through a conditional Cre targeting floxed ECPR alleles. The Cre

excision is imperfect, leading to EPCR^{lo} mice rather than complete knockouts. EPCR^{lo} mice expressing zyPC were protected against metastasis compared to PBS control EPCR^{lo} mice, and at similar levels of protection seen in Cre-less floxed EPCR mice expressing zyPC (**Figure 5.3C**). Interestingly, untreated EPCR^{lo} mice generated from homozygous floxed alleles (KO), EPCR^{lo} mice generated from a single floxed allele and a wild type allele (Het), and Cre-less floxed EPCR mice (F/F) all had similar levels of metastasis (**Figure 5.3D**). This is surprising given previously published data that overexpression of EPCR decreases tumor metastasis; however, it is possible that enough EPCR remains as to not exacerbate tumor progression.

zyPC anti-metastatic properties are not mediated through PAR-4

Murine PAR-4 is a platelet PAR that, when knocked out, reduces the number of tumor metastasis. Thus, it is possible that zyPC binding could mediate a similar effect. Therefore, we explored the effect of zyPC on metastasis in PAR-4 knockout mice, but found that, as with PAR-1 knockouts, zyPC was equally effective in preventing metastasis with or without PAR-4 (**Figure 5.4**).

zyPC anti-metastatic properties are not mediated through β_1/β_3 integrin binding

mzy/aPC has a QGD putative β_1/β_3 integrin binding site at amino acids 220-222 (RGD in haPC); when bound, aPC prevents neutrophil adhesion and migration²⁶¹. Such a mechanism could easily be hijacked by tumor cells, and zy/aPC binding could block the needed receptors for tumor cell adhesion and invasion. Therefore, we made a QGE

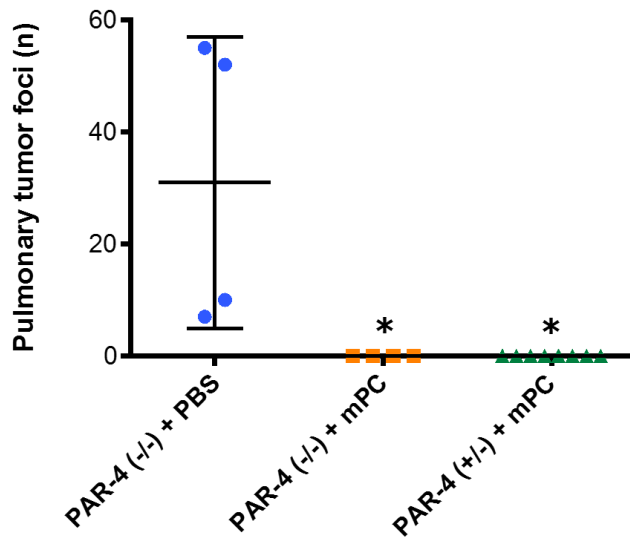


Figure 5.4 zyPC anti-metastatic properties are not dependent on interaction with platelet PAR-4. PAR-4 knockout mice were administered 5×10^{13} vg/kg AAV8-mzyPC (orange) or PBS (blue) and compared to PAR-4 heterozygous littermate controls that received 5×10^{13} vg/kg AAV8-mzyPC (green) intravenously three weeks prior to intravenous injection of 2.5×10^5 murine B16F10 cells. Lungs were removed 21 days later and pulmonary tumor foci visible to the naked eye were quantified. Significance is comparing the treated groups to the PBS control group. Horizontal bars indicate mean \pm SD. * $p < 0.05$.

mutant (zyPC-D222E) shown to ablate binding in haPC. However, zyPC-D222E did not have reduced anti-metastasis abilities (**Figure 5.5**).

Human zyPC is not as protective in a murine model of metastasis as murine zyPC

Human and murine protein C share 69% homology, but human aPC has 6 times less anticoagulant activity and 10 times less neuroprotective effects, which likely require the anti-apoptotic properties of aPC²⁶². This reduced functionality of hzyPC in mice *in vivo* is evident in the protection conferred in this B16F10 metastasis model (**Figure 5.6**).

While still highly protective, the hzyPC mice formed an average of 20 times as many tumors as the mzyPC mice.

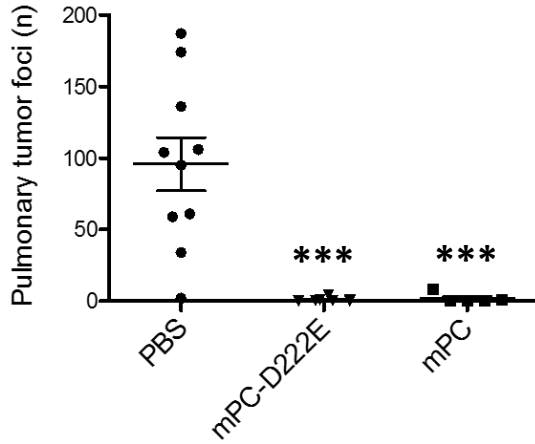


Figure 5.5 zyPC anti-metastatic properties are not mediated through the QGD putative β_1/β_3 integrin binding site on PC. Mice were administered 5×10^{13} vg/kg AAV8-mzyPC (squares), -mzyPC-D222E (triangles), or PBS (blue) intravenously three weeks prior to intravenous injection of 2.5×10^5 murine B16F10 cells. Lungs were removed 21 days later and pulmonary tumor foci visible to the naked eye were quantified. Significance is comparing the treated groups to the PBS control group. Horizontal bars indicate mean \pm SD. *** $p < 0.001$.

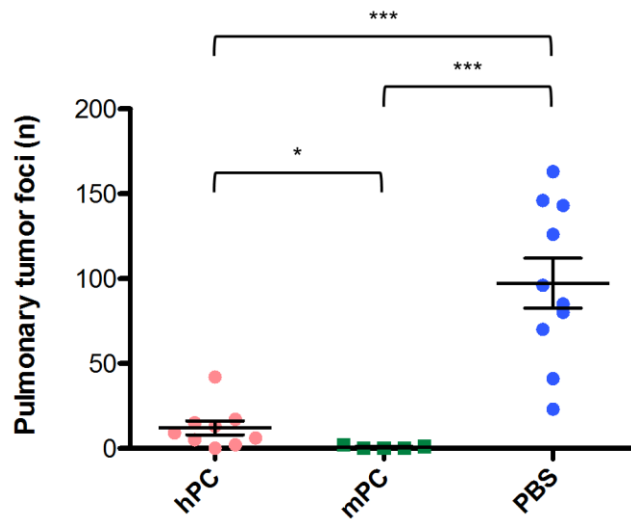
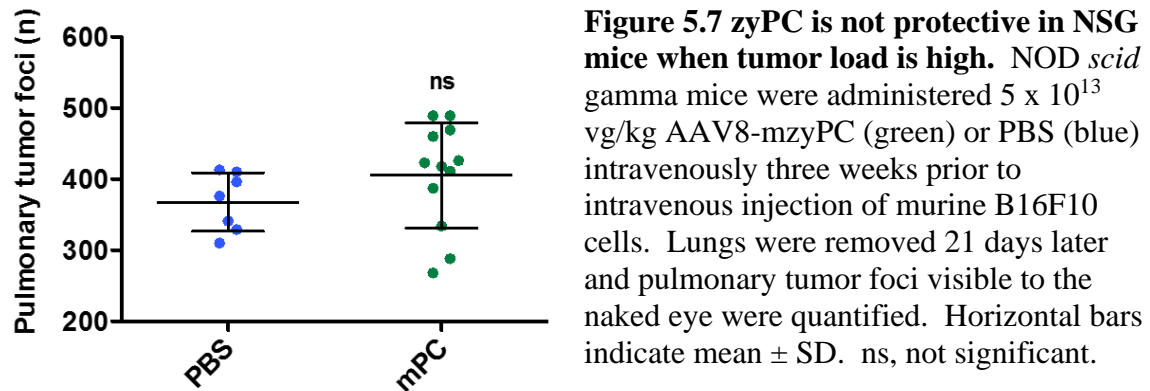


Figure 5.6 Human zyPC is not as protective as murine zyPC in a murine model of metastasis. Mice were administered 5×10^{13} vg/kg AAV8-mzyPC (green), -hzyPC (pink), or PBS (blue) intravenously three weeks prior to intravenous injection of 2.5×10^5 murine B16F10 cells. Lungs were removed 21 days later and pulmonary tumor foci visible to the naked eye were quantified. Horizontal bars indicate mean \pm SD. * $p < 0.05$; *** $p < 0.001$.



NSG mice are not protected against metastasis with zyPC when tumor load is high

In order to determine if the anti-metastatic properties of zyPC are dependent upon direct interaction with immune cells, we utilized the NOD *scid* gamma mouse line. NSGs are lacking mature B and T cells, NK cells, and complement and have defective dendritic cells and macrophages. As would be expected, these mice could not tolerate the normal dose of 2.5×10^5 B16F10s, and so were given a lower dose of 6.5×10^4 . Despite this lower dose, the tumor load in all animals was still very high, and there was no difference seen in the animals expressing zyPC compared to the control animals (**Figure 5.7**).

zyPC-mediated anti-metastasis protection is not limited to B16F10 melanoma cells

While there is an apparent lack of direct effect on B16F10 cell growth (**Figure 5.1A**, **5.2**), adhesion (**Figure 5.1B**), and transmigration²⁵², it is possible that the anti-metastatic effect is cancer cell type specific, either by a yet undiscovered direct tumor effect or because of differing tumor cell adhesion and/or extravasation receptors. Thus, we explored whether zyPC would be protective in a model of metastatic breast cancer.

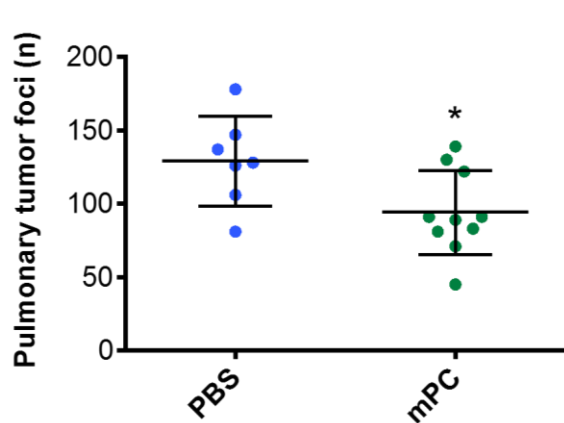


Figure 5.8 zyPC is protective against metastasis in the 4T1 breast cancer model. Mice were administered 5×10^{13} vg/kg AAV8-mzyPC (green) or PBS (blue) intravenously three weeks prior to subcutaneous injection of 1×10^4 murine 4T1 cells. As mice reached experimental endpoints related to primary tumor size and animal wellness, they were sacrificed and lungs and primary tumors removed. Pulmonary tumor foci visible to the naked eye were quantified. Horizontal bars indicate mean \pm SD. * $p < 0.05$.

Unlike with the B16F10 melanoma model, 4T1 cells are subcutaneously injected to form a primary tumor on the animal's flank, which becomes metastatic upon reaching a large enough size. That primary tumor will then continuously seed new metastases unless resected, making it a highly aggressive model of metastasis. As such, the pulmonary tumor load in these animals was quite high. However, we did still see a slight reduction in the number of metastases in the lungs of the zyPC treated animals (**Figure 5.8**).

Importantly, there was no difference in the size or progression of the primary tumors between the groups (data not shown).

IV. Discussion

Our *in vitro* and primary tumor work demonstrates that zyPC does not have a direct effect on B16F10 cell growth, nor does it impact B16F10 adhesion to proteins of the basement membrane. This is comparable to previous work that demonstrated aPC could only

prevent B16F10 cell adhesion to and transmigration across an endothelial cell monolayer when the endothelial cells—not the B16F10s—were pre-treated with aPC²⁵².

Collectively, these data suggest that the anti-metastatic properties of zy/aPC are limited to affecting the host, not the tumor cells.

This, in conjunction with the data shown in Chapter 4, further implies that the mechanism of protection is likely to be via competitive blocking of an endothelial receptor or membrane protein that prevents B16F10 tumor cell adhesion and/or extravasation. Unfortunately, the potential receptors are endless, so we chose to focus on receptors already identified as putative zy/aPC binding²⁵⁴.

The first receptor we investigated was the endothelial protein C receptor, as this had already been demonstrated as being involved in the zy/aPC anti-metastatic pathway²⁵². Overexpression of EPCR in Tie2-EPCR transgenic mice had led to a massive reduction in the number of pulmonary tumor foci formed after B16F10 cell injection. However, we demonstrated through three different methods that zyPC-based protection is not dependent upon EPCR binding, suggesting that the overexpression of EPCR is having a different effect than previously thought. One possible explanation is that with more EPCR available for binding, plasma levels of zyPC may go up, and it is these increased levels that facilitate reduced metastasis.

We also attempted to explore the necessity of the immune system and white blood cell receptors on the anti-metastatic properties of PC. We successfully demonstrated that binding to β_1 and/or β_3 receptors is not required, but our results with the NSG mice were less clear. While we did not see zyPC-mediated protection in these mice, it is possible that the high tumor load exceeded the ability of zyPC to modulate metastasis. Thus, the experiment should be repeated with even lower B16F10 cell doses.

Despite that fact that previous work has shown involvement of PAR-4 in B16F10 metastasis, we did not see a loss of ability of zyPC to protect against metastasis in PAR-4 knockout animals. This is one of just a couple of platelet-related receptors that zy/aPC is believed to bind, the other two being platelet receptors on the endothelium: MAC-1 and apoER2 (also known as LRP8). LRP8 was recently shown to be involved in B16F10 and human melanoma cell extravasation through mutual apoE binding by apoER2 on the vessel wall and LRP1 on the cancer cells. Should PC somehow interfere with this apoER2/apoE or apoE/LRP1 binding, it is possible that this is the mechanism through which the protein C pathway prevents metastatic cancer progression.

We also showed that hzyPC has reduced anti-metastatic effects compared to mzyPC in the murine model (approximately 20 times less), although the number of tumors in the hzyPC-expressing mice compared to untreated animals still reflected an 88% reduction in the number of metastasis. This reduced efficacy of hzy/aPC is paralleled in the reduced anticoagulant function, which is thought to be due to reduced PS binding, seen *in vivo* in

mice and *in vitro* in murine plasma, as well as in the reduced neuroprotective effects seen in ischemic stroke, which again is thought to be due to reduced receptor binding. Thus, the loss of anti-metastatic properties with species specificity further supports the idea that zy/aPC protection against cancer progression requires protein-protein interaction, rather than, for example, catalytic function.

Finally, we demonstrate that the protection seen in our work and the previously published work of others^{252, 231} utilizing B16F10s is not restricted to the melanoma cancer cell model. This provides hope that, should a pharmacologic intervention ever result from these studies, it could potentially benefit a wider range of patients than just those with cancers of the skin. Ultimately, though, for such a product to reach the market, more must be understood about the mechanism of action of the protein C pathway's ability to prevent cancer progression by blocking tumor metastasis.

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

The field of gene therapy is currently undergoing a resurgence after falling out of favor with the death of Jesse Gelsinger in 1999 and issues with childhood leukemia in the early 2000s. In 2012, Glybera, an AAV1 expressing lipoprotein lipase, was approved in Europe, making it the first gene therapy product licensed in the Western world. There have been a number of additional promising and successful gene therapy clinical trials in the last few years, from hemophilia to Leber's congenital amaurosis to cancer, and it is expected that a number of new products will be joining Glybera in the near future.

This work demonstrates that gene therapy with coagulation protein transgenes has the potential to treat hemophilia A (Chapter 2) and B (Chapter 3) patients currently excluded from clinical trials for inhibitors by tolerization and immune modulation following liver-directed AAV gene therapy, as well as modulating cancer progression through inhibition of metastasis (Chapters 4 and 5).

Hemophilia, gene therapy, and the immune system

Hemophilia has a nearly 15-year history of gene therapy, with promising results in the last few years following liver-directed gene therapy for HB with AAV8²⁶³. However, even those promising trials have required courses of prednisolone in most patients

receiving efficacious vector doses in order to prevent anti-AAV capsid CD8⁺ cytotoxic T cells from destroying the transduced hepatocytes and resulting in loss of transgene expression. In Chapter 3, we demonstrated that vector doses for HB can be dramatically lowered with no loss of FIX activity by utilizing the naturally occurring, hyper-functional FIX-Padua as the transgene, potentially allowing for vector doses low enough to prevent immune activation and avoid the use of steroids. Importantly, we demonstrated that this increased specific activity does not increase the risk of either thrombophilia—even in a dog with nearly 300% FIX activity—or transgene-product immunogenicity, despite the point mutation. This indicates that FIX-Padua is as safe as the wild type FIX transgene that has been used in numerous clinical trials with no adverse effects to date, and that moving FIX-Padua into people is a reasonable proposal. One such clinical trial with AVV-FIX-Padua is currently underway and another is in the planning stages. Hopefully the results of the preclinical work done by our lab and others with this novel transgene will be mirrored in human subjects, greatly increasing the efficacy and safety of HB gene therapy.

While our lab is currently working to determine the mechanism responsible for the heightened specific activity of FIX-Padua, the work shown in Chapter 3 has been a proof-of-principle that modifications to the transgene that result in higher expression or activity might be utilized to overcome the vector-dose-dependent, anti-capsid immune response. For example, if a corresponding modification can be made in FIX's cofactor, FVIII, that could have profound impacts for HA gene therapy as well. Additionally, our lab is

exploring other FVIII variations seen in cFVIII that results in higher secretion compared to hFVIII. As with the studies done here, any such modifications must be rigorously tested for safety, especially with relation to immunogenicity.

In all clinical trials to date, hemophilia patients with inhibitors have been excluded. However, as shown in Chapters 2 and 3, liver-directed AAV gene therapy has the ability to induce tolerance to the transgene, even in the context of a pre-existing, ongoing immune response to that transgene. It is possible, therefore, that the risk-benefit analysis for inclusion in a gene therapy trial might actually be slanted toward preferentially including inhibitor patients, who otherwise face a grueling and expensive ITI protocol. Beyond the world of hemophilia, many diseases could potentially benefit from AAV/liver-driven transgene tolerization. For example, autoimmune diabetes has been prevented in NOD mice following AAV expression of the beta-cell autoantigen glutamic acid decarboxylase 65 peptide GAD₍₅₀₀₋₅₈₅₎ from the liver²⁶⁴. A number of additional autoimmune diseases such as autoimmune hemolytic anemia, multiple sclerosis, autoimmune thrombocytopenic purpura, and rheumatoid arthritis have known autoantigen targets that do not necessarily require nor would they necessarily benefit from straight transgene replacement. In these instances, liver-restricted expression and resulting retolerization could halt disease progression and potentially even prevent disease entirely.

Gene therapy induced tolerance can also benefit a group of patients with genetic diseases, such as metabolic disorders like Pompe disease, where enzyme replacement therapies (ERT) currently on the market are ultimately inhibited by the development of neutralizing antibodies to the infused protein. While this scenario is similar to the development of inhibitors in hemophilia patients, up to 90% of juvenile patients will develop antibodies to ERT, and there is no standard ITI protocol for Pompe disease. In the few instances where a case is successfully treated it is cause for a New England Journal of Medicine paper²⁶⁵. Unfortunately, it takes extremely high levels of enzyme to ameliorate the disease phenotype in Pompe—well outside the range currently achievable with gene therapy—which would traditionally make it a poor candidate for current gene therapy practices. However, these patients would benefit highly from ITI and, as such, AAV liver-directed gene therapy, for the sole purpose of tolerizing the patient to the pharmacologic product as this would keep their ERT efficacious.

The question remains how, exactly, AAV expression in the liver can induce and maintain tolerance to the transgene. As was discussed at length in Chapter 1, it is likely a multifaceted immune response, with data in murine models pointing to anergy, T cell deletion, and Treg induction. In Chapter 2 we showed that in dogs with pre-existing inhibitors, CD4⁺CD25⁺FoxP3⁺ T cells expanded 1 to 2 weeks after AAV8-FVIII vector administration and immediately prior to disappearance of FVIII-specific antibodies. This expansion was not seen in dogs without pre-existing inhibitors and was delayed in a dog that developed a transient inhibitor after gene therapy. While identification of these T

cells as FVIII-specific Tregs is impossible given current technologies, the timeline in conjunction with what is known from the aforementioned previous experience in transgenic mice suggests that these could be FVIII CD4⁺ induced Tregs.

Furthermore, in Chapter 3 we saw a noticeable lack of IL-2, a sign of Treg activity, in the FIX inhibitor dog following AAV8-FIX-Padua gene therapy, despite an ongoing, pro-inflammatory, Th2 immune response as indicated by elevated levels of GM-CSF, IL-15, IL-6, and IL-18. Additionally, IL-10—a classic Treg tolerogenic cytokine—levels were highest 2-4 weeks after vector administration (around the time we saw the increased frequency of CD4⁺CD25⁺FoxP3⁺ T cells in the FVIII inhibitor model and correlating with the elimination of anti-FIX neutralizing antibodies). IL-10 also began to rise again immediately following challenge with FIX concentrate, suggesting further suppression of an anti-FIX immune response was required.

Interestingly, IFN- γ also peaked with IL-10. IFN- γ is traditionally considered to be a Th1 and CD8⁺ T cell cytokine; however, the lack of correlating IL-2 and TNF- α is surprising were that the case. While it is possible this is indicative of an anti-FIX cytotoxic T cell response, this is further unlikely given that inhibitors are traditionally thought of as Th2 and antibody driven. Instead, there is some indication in the literature (see Chapter 2 discussion) that conversion of CD4⁺ T cells into induced Tregs in the periphery requires and/or produces IFN- γ . If this is the case here, the dual peaks at weeks 2-4 and

immediately following challenge with FIX concentrates further supports the idea that Tregs are induced following AAV administration and again following challenge.

TNF- α levels were low throughout and similar cytokine profiles were not seen in two non-inhibitor dogs, indicating that this is not simply an anti-vector immune response.

Further canine immunology reagents must be developed and additional studies done to fully understand the mechanisms by which AAV-directed transgene expression in the liver is able to induce tolerance in the context of a pre-existing and ongoing immune response against the transgene. Such understanding would allow researchers to more fully comprehend the risks and rewards involved in utilizing gene therapy for ITI as well as potentially provide translatable practices to current recombinant protein-based ITI protocols.

Activated and zymogen protein C and cancer metastasis

While much about the protein C pathway has been well characterized, there is just as much that remains hotly debated. Even the well-established anticoagulant and cytoprotective functions have lingering mechanistic questions (Does aPC really cleave FVIIIa or PAR-1 *in vivo*?). Much less is understood about any potential functionality of zyPC, which is often ignored in the literature, considered to be just a proenzyme waiting to be activated. In this sea of ambiguity, it was discovered that aPC has the ability to reduce tumor metastasis, so researchers endeavored to determine the mechanism of action

by focusing on the most well understood and the most likely to be involved aPC functions: anticoagulation and cytoprotection.

While there is overwhelming evidence that coagulation enhances metastasis and supporting data suggesting that inhibiting coagulation prevents metastasis, Van Sluis *et al.*²⁶⁶ showed compelling data that inhibiting the anticoagulant function of endogenous aPC with an antibody that selectively binds aPC rather than zyPC but that left the cytoprotective function intact did not worsen the number of pulmonary metastatic lesions formed²⁶⁷. Conversely, knocking out both the anticoagulant function and cytoprotective function via an antibody that prevents PC binding to cell membranes (likely by blocking binding to EPCR) increased the number of metastatic tumors formed. The authors interpreted this as an indication that anticoagulant function is not necessary for the anti-metastatic properties of aPC, and that the cytoprotective function is. Furthermore, they were able to reduce the number of tumors formed in animals treated with the second antibody back to baseline levels by using an S1P₁ agonist, demonstrating that aPC's protective properties function through the PAR-1/SphK-1/S1P₁ receptors pathway. In addition, Bezuhly *et al.*²⁶⁸ showed that overexpressing EPCR in transgenic mice decreased the number the metastases, though they did not explore how EPCR overexpression affected zy/aPC levels. Collectively, these two papers suggest that endogenous aPC protects against metastasis via the PAR-1-mediated cytoprotective effect.

However, this was not what we saw in our model. Knocking out the cytoprotective effect (E149A) did not diminish a/zyPC's anti-metastatic properties, nor did knocking out PAR-1 in the mice. Additionally, the 2A, 3A, and 5A mutants, which retain cytoprotective function, had reduced abilities to protect against metastasis. This suggests that *endogenous* levels of zy/aPC cytoprotection is important in modulating tumor progression, but that additional cytoprotection is of no benefit for preventing tumor metastasis. Alternatively, it is possible that the antibody used by Van Sluis *et al.*²⁶⁷ blocks more than just the cytoprotective and anticoagulant effect of zy/aPC.

We also demonstrated that, despite previously published work to the contrary, EPCR binding is not necessary for the anti-metastatic effect (blocking antibodies, EPCR^{lo} mice and Gla domain swaps), suggesting that perhaps the Tie2-EPCR transgenic mice are protected due to another mechanism, such as an increase in zyPC levels in response to more receptor available for binding.

Finally, we showed that aPC is not working through its anticoagulant function (aPC-L38D, zyPC, zyPC-R15Q, zyPC-S195A, zyPC-R15QS195A), even though it is known that blocking coagulation in mice can reduce metastasis (see Chapter 1 for thorough discussion).

After eliminating the most likely mechanisms of protection, we began examining putative PC-binding receptors. $\alpha_M\beta_2$ and β_1/β_3 integrins are involved in white blood cell adhesion,

while apoER2 and GP1b α are involved in platelet adhesion²⁶⁹. PAR-4 is also a platelet receptor. We have eliminated the involvement of PAR-4 (knockout mice) and β_1/β_3 integrins (zyPC-D222E). NSG mice showed no reduction in tumor metastasis with treatment, but the tumor load was extremely high and may simply have been too high for treatment to be effective. Going forward, the NSG mice experiment should be repeated with an even lower dose of B16F10s to further determine if there is involvement of the immune system. Antibodies and knockout mice for GP1b α and LRP8/apoER2 knockout mice are available and should be utilized.

Throughout this work we refer to the ability of the protein C pathway to modulate tumor progression as affecting metastasis. The lack of inhibition of primary tumor and *in vitro* cell growths support this, as does work by Bezuhly *et al* that demonstrated reduced transendothelial migration following B16F10 exposure to rhaPC²⁶⁸. Interestingly, we did not show reduced adhesion of B16F10s to collagen, fibrinogen, or Matrigel, suggesting that the loss of adhesion seen by Bezuhly *et al* was due to blocking something on the bEnd.3 cells rather than on the B16F10s. This had been suggested by those authors as well, as pre-treating B16F10s with rhaPC did not prevent binding to bEnd.3 monolayers like pre-treating the bEnd.3 cells did. Likewise, they also showed that pre-treatment of the bEnd.3 cells but not the B16F10 cells was able to reduce transmigration across the bEnd.3 monolayer and transwell membrane. Collectively, these data suggest that the ability of zy/aPC to prevent cancer progression is truly through limiting the metastatic pathway by acting on the host rather than directly on the tumor cells.

Previously, the potential of using aPC to combat cancer progression seemed unlikely given its extremely short half-life (approximately 15 minutes) and the removal of Xigris, recombinant human activated protein C intended for use in sepsis treatment, from the market after failed efficacy and bleeding risks. However, the zymogen has a 6 hour half-life and could be further extended as progress has been made in the hemophilia market with modifications such as PEGylation to greatly increase the half-life of clotting factors. Additionally, zyPC, and especially zyPC-R15QS195A, would not have the same risk of bleeds as aPC. Interestingly, injections of recombinant human zyPC into septic, but otherwise healthy, volunteers at fold levels over baseline similar to those seen here following gene therapy caused no adverse effects²⁷⁰. It is possible that treatment of patients with cancers known to be metastatic, especially at the time of primary tumor resection, could have a disease progression benefit for patients.

While recombinant zymogen PC can be produced and marketed, if the mechanism of action of metastasis prevention can be found it is possible that a longer acting small molecule or antibody could be produced. In the interim, it is also possible that gene therapy with AAV to express zyPC-R15QS195A could be as protective in human subjects as it is in animal models. However, potential side effects due to long-term competition with wild type zyPC should be explored, and any trials conceived should target patients with the highest risk of mortality due to cancer types with known tendencies to quickly metastasize so as to ensure a proper risk-benefit ratio.

Closing Remarks

The potential applications for gene therapy seem endless, and range dramatically from life-saving neonatal therapies to address fatal fetal anomalies to elective treatments to correct male-pattern baldness. As this dissertation has demonstrated, gene therapy has a place not only in treating monogenetic inherited disorders by solely replacing a mutant gene but also in manipulating the immune system and in appropriating normal physiologic processes to new ends.

CHAPTER 7

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II. Chapter 2

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APPENDIX A
ABBREVIATIONS

a, activated

aPC, activated protein C

aPTT, activated partial thromboplastin time

AAV, adeno-associated virus or adeno-associated viral vectors

BDD, B-domain deleted

BU, Bethesda units

c, canine

CRM, cross reacting material

EGF, epidermal growth factor

ELISA, enzyme-linked immunosorbent assay

EPCR, endothelial protein C receptor

ERT, enzyme replacement therapy

F, factor

GM-CSF, granulocyte macrophage colony-stimulating factor

h, human

HA, hemophilia A

hAAT, human alpha-1 anti-trypsin

HB, hemophilia B

HC, heavy chain (of FVIII)

HD, high dose

IFN, interferon

IL, interleukin

ITI, immune tolerance induction

ITR, inverted terminal repeat

iTreg, induced Treg

LC, light chain (of FVIII)

LD, low dose

m, murine

MD, mid dose

MS-1, murine Mile Sven 1 endothelial cells

NAb, neutralizing antibody

NF κ B, nuclear transcription factor κ B

NK, natural killer (cell)

NSG, NOD *scid* gamma mice

PAR, protease activated receptor

PBMCs, peripheral blood mononuclear cells

PBS, phosphate buffered saline

PS, protein S

r, recombinant

S1P, sphingosine-1-phosphate

S1P₁, S1P receptor-1

SphK-1, sphingosine kinase-1

TAT, thrombin-antithrombin complex

TNF, tumor necrosis factor

TBG, human thyroxine binding globulin

TF, tissue factor

TM, thrombomodulin

TEG, thrombelastography

UAB, University of Alabama at Birmingham

UNC, University of North Carolina (at Chapel Hill)

VTE, venous thromboembolism

WBCT, whole blood clotting time

zyPC, zymogen protein C

APPENDIX B

DOGS

HA dogs

K01, pre-existing cFVIII inhibitors from UNC, treated with 2.5×10^{13} vg/kg of AAV8-cFVIII-LC and 2.5×10^{13} vg/kg AAV8-cFVIII-HC i.v.

K03, pre-existing cFVIII inhibitors from UNC, treated with 2.5×10^{13} vg/kg of AAV8-cFVIII-LC and 2.5×10^{13} vg/kg AAV8-cFVIII-HC i.v., FVIII coverage at the time of vector administration

L44, pre-existing cFVIII inhibitors from UNC, treated with 2.5×10^{13} vg/kg of AAV8-cFVIII-LC and 2.5×10^{13} vg/kg AAV8-cFVIII-HC i.v.

L51, inhibitor prone from UNC, developed transient inhibitors, treated with 2×10^{13} vg/kg of AAV8-cFVIII-BDD single chain i.v.

Linus, inhibitor prone from UNC, treated with 6.0×10^{12} vg/kg of AAV8-cFVIII-HC and 6.0×10^{12} vg/kg AAV8-cFVIII-LC i.v.

M06, inhibitor prone from UNC, treated with 4×10^{13} vg/kg AAV8-cFVIII-BDD single chain i.v.

Wembley, pre-existing hFVIII inhibitors from Queen's University, large anamnestic response, treated with 2.5×10^{13} vg/kg of AAV8-cFVIII-LC and 2.5×10^{13} vg/kg AAV8-cFVIII-HC i.v.

HB dogs

Trex, inhibitor-prone from UAB, treated with 3×10^{12} vg/kg AAV8-cFIX-Padua i.v.

Wick, inhibitor prone from UAB, treated with 1×10^{12} vg/kg AAV8-cFIX-Padua i.v.

Wiley, pre-existing hFIX inhibitors from UAB, treated with 3×10^{12} vg/kg AAV8-cFIX-Padua i.v.